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13. ABSTRACT (Maximum 200 Words)

The transcription factor, C/EBPbeta, is critical for growth and differentation of the mammary gland. Three forms of C/EBPbeta are expressed in cells through alternative translation of the C/EBPbeta mRNA. C/EBPbeta-1 and –2 activate transcription; C/EBPbeta-2 is a truncated form that represses transcription. Based upon their distinct expression patterns in normal and transformed cells as well as functional studies, we propose that C/EBPbeta-1 and –3 (or both) will be potent anti-tumor therapies when used either singly or in combination by suppressing the expression of genes which drive the cell cycle (C/EBPbeta-3) and/or reactivating a differentiation program (C/EBPbeta-1). The purpose of this study is to develop non-replicating murine retroviral and adenoviral vectors carrying genes selectively encoding either C/EBPbeta-1 or –3. These vectors will be used to efficiently re-express C/EBPbeta-1 and/or overexpress C/EBPbeta–3 in breast cancer cells lines and evaluate the growth potential of the cells. We have successfully generated murine retroviral vectors encoding C/EBPbeta-1 and C/EBPbeta-3 as well as an adenovirus encoding C/EBPbeta-3. We have infected breast cancer cell lines with each of these viruses and documented nuclear expression of C/EBPbeta-1 and –3 by both immunohistochemistry and immunoblotting. Studies on the growth potential of the infected cells are confidential and will be presented in the annual report.

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Transcription factor, viral vector, anti-tumor therapy, C/EBPbeta

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INTRODUCTION

Gene deletion studies have shown that the transcription factor, C/EBPbeta, is critical for growth and differentiation of the mammary gland. Both increased epithelial cell proliferation in early pregnancy and differentiation at late pregnancy stages are strongly impaired in the absence of C/EBPbeta. Furthermore, C/EBPbeta null mice fail to lactate. Three forms of C/EBPbeta can be expressed in cells through alternative translation of the C/EBPbeta mRNA at one of three in frame methionines. C/EBPbeta-1 and C/EBPbeta-2 activate transcription; C/EBPbeta-3 is a truncated form that represses transcription. Because they are close in size, functional differences between C/EBPbeta-1 and -2 have never been investigated. Using an antibody we developed that is specific for C/EBPbeta-1, we made the novel discovery that C/EBPbeta-1 is expressed only in normal human mammary epithelial cells (HMECs) and ductal secretory epithelial cells from human breast milk, but not in any breast cancer cell lines we have examined (1). Moreover, C/EBPbeta-2 is sequestered in the cytoplasm in normal HMECs, but constitutively activated in the nucleus in breast cancer cells (1). Finally, C/EBPbeta-3 is expressed at high levels in growing HMECS, whereas some breast cancer cell lines lack this repressor (1). Based upon their expression pattern and other functional studies, we propose that C/EBPbeta-3 or 1 (or both) will be potent anti-tumor therapies when used either singly or in combination to inhibit tumor cell growth by suppressing the expression of genes which drive the cell cycle (C/EBPbeta-3) and/or reactivating a differentiation program (C/EBPbeta-1). To test this hypothesis we are developing non-replicating murine retroviral vectors and/or adenoviruses that carry genes selectively encoding either epitope-tagged C/EBPbeta-1 or C/EBPbeta-3. After infection with these viruses, we will evaluate the growth potential of breast cancer cells lines overexpressing either epitope-tagged C/EBPbeta-3 or epitope-tagged C/EBPbeta-1, or both.

BODY

TASK 1: To generate retroviral vectors encoding his-tagged C/EBPbeta-3 (in hand) or C/EBPbeta-1 (months 1-3; status – completed.)

Site directed mutagenesis of the C/EBPbeta cDNA was accomplished using a mutant oligonucleotide as described in Eaton et al. (1) in which the 2nd in frame methionine was mutated to valine and a perfect Kozak sequence was created around the first ATG to ensure selective expression of C/EBPβ-1. The resultant C/EBPbeta-1 cDNA was tested for exclusive expression of his-tagged C/EBPbeta-1 by Western blotting with the T7 his tag antibody (1) prior to transfer to the LZRSpBMN-Z retroviral vector (2,3).

TASK 2: To produce recombinant, helper free C/EBPbeta-1 or C/EBPbeta-3 retroviral stocks (months 1-6; status – completed.)

To obtain non-replicating retrovirus we transfected the LZRS-based his-C/EBPbeta-3, his-C/EBPbeta-1, or the control beta-gal construct into a 293T-based amphotropic packaging cell line termed ϕnx-ampho. The ϕnx-ampho packaging cell line was specifically developed by G. Nolan to produce high titer, helper free recombinant retrovirus (2,3). Three weeks prior to performing transfections, ϕnx-ampho cells were reselected in the presence of diptheria toxin and hygromycin B to increase envelope and gag-pol expression. Packaging cells were then transfected by standard CaPO4 procedures, and viral supernatants were harvested at 48hrs post-transfection, clarified, and stored frozen at -800C. Cells were then trypsinized and replated in medium containing puromycin (to select for episomal maintainance of the LZRS-based construct) . Upon reaching 70% confluence, cells were placed in puromycin-free medium for 24 hrs prior to harvesting virus as before. This procedure was carried out for production and collection of viral stocks for up to 3 weeks post-transfection. We performed this procedure with both the control LZRS-β-gal or LZRS-hisC/EBPbeta-1 and -3 constructs and successfully obtained non replicating

retroviral stocks for each. However, when infecting cells with these chimeric retroviruses, we soon found that although beta-galactosidase assays were easy to perform, detecting the epitope-tagged C/EBPbeta proteins by immunofluorescent staining was slow and laborious. Therefore, we modified the hybrid retroviral vector to aid in identifying and quantifying the number of infected cells. We inserted an internal ribosome entry sequence (IRES) linked to a green fluorescent protein (GFP) coding region downstream of the C/EBPβ isoform. Because both C/EBPβ and GFP are expressed from a single viral mRNA the correspondence of coexpressing cells is essentially 100%. Therefore GFP can be used as a marked for tagged-C/EBPβ expressing cells. Both hisC/EBPβ-1-IRES-GFP and hisC/EBPβ-3-IRES-GFP retrovirus were produced, along with the control IRES-GFP retrovirus. Titers ranged between 10^6 and 10^7 infectious units per ml. The presence of GFP also enables us to sort the infected cell population by FACS if desired.

TASK 3: To generate a recombinant adenovirus encoding epitope-tagged C/EBPbeta-3 (months 4-6; status – completed.)

We cloned a cDNA for epitope-tagged C/EBPbeta-3 into the pGEM-RecA vector. Recombinant adenoviral stocks were prepared from this construct in 293 cells by Dr. Rosie Sears in the laboratory of Joe Nevins and generously provided to us. Titers ranged between 10^{10} and 10^{11} infectious units per ml. The recombinant C/EBPbeta-3 adenovirus also encodes for GFP as a marker protein as well

TASK 4: To infect a representative panel of breast cancer cell lines with hisC/EBPbeta-3, hisC/EBPbeta-1 or control beta-gal retroviruses and analyze growth potential of the infected cells (months 6-24; status – ongoing).

Progress on task 4 will be divided into two sections, addressing either hisC/EBPbeta-1 or –3, respectively.

C/EBPbeta-1

In year 1, we demonstrated that the breast cancer cell line, MDA 231, can be infected with hisC/EBPbeta-1-IRES-GFP retrovirus. hisC/EBPbeta-1 expression in the cells as monitored by immunofluorescent staining with T7tag antibody was seen to be primarily nuclear, as expected. In year 2, we also successfully infected another breast cancer cell line, MDA 435, with hisC/EBPbeta-1-IRES-GFP retrovirus. We prepared whole cell extracts from both hisC/EBPbeta-1-IRES-GFP and IRES-GFP infected MDA 231 and MDA 435 cells as well as uninfected cells for analysis of protein expression levels by immunoblotting. (Note: Because we have included GFP as a marker protein in all of our retroviral constructs, we routinely use a chimeric retrovirus expressing just GFP as a control, rather than beta-gal.). Retroviral infection results in a high level of hisC/EBPbeta-1 expression in the cells, relative to the endogenous level of C/EBPbeta2 (see Figure 1). The level of hisC/EBPbeta-1 expression in the breast cancer cells is similar to the level of endogenous C/EBPbeta-1 expression observed in normal prim;ary human mammary epithelial cells (HMECs).

MDA-231 cells infected with hisC/EBPbeta-1-IRES-GFP retrovirus or IRES-GFP retrovirus were sorted by FACS. No differences were observed in the proportion of cells in S phase (Figure 2) and the doubling time was not affected by C/EBPbeta-1 reexpression in these cells (Figure 3). However, we obtained an interesting result from the colony assay, in which C/EBPbeta-1 infected or control cells were plated at approximately 800 cells per 60mm dish. MDA 231s infected with C/EBPbeta-1 formed colonies of fewer cells than MDA 231s infected with GFP only (Figure 4). It is possible that introduction of C/EBPbeta-1 into these cells causes contact inhibition and decreases their ability to grow on top of one another. To address this possibility, in year 02 we examined the ability of the cells to grow in matrigel, a synthetic basement membrane prepared from Englebreth-Holm-Swarm (EHS) tumor. MDA 231 cells form a characteristic highly branched, spindle growth pattern when placed in the matrigel environment; in contrast, cells which maintain their epithelial characteristics and are contact-inhibited form acinar structures (4).

As shown in Figure 5, MDA-231 cells reexpressing C/EBPbeta-1 no longer display the branching, stellate morphology of the control MDA 231 cells expressing GFP only. Instead, they grow with a fused, spherical morphology. These differences resemble those seen between invasive and non-invasive breast cancer cells lines (4). The fused, spherical morphology is typical of non-invasive cell lines such as MCF-7, whereas highly invasive, metastatic cell lines (MDA 231, MDA 435) exhibit a branching morphology as mentioned above. To confirm these studies tested the highly invasive cell line, MDA 435 infected with hisC/EBPbeta-1-IRES-GFP retrovirus or IRES-GFP retrovirus. The invasive, branching growth of the untreated MDA 435 cells, or GFP only expressing cells, was completely blocked by C/EBPbeta-1 expression (Figure 6). As an additional control, expression of the C/EBPbeta-2 isoform, which is the transactivator isoform found in breast cancer cell lines and acquired by invasive human mammary carcinomas (1), had no effect on the growth pattern of MDA 435 cells in matrigel. These studies suggest that although the transformed MDA 231 and 435 cells do not form normal acinar structures, C/EBPbeta-1 expression may have a significant effect on reducing cell motility and invasive capacity.

Because of the morphologic changes we observed in the C/EBPbeta-1 expressing breast cancer cell lines, we decided to investigate the effect of this protein on behavior in an in vitro invasion assay. In this assay, cells must degrade a uniform barrier of matrigel and traverse a porous filter in response to a chemotactic gradient in a modified Boyden chamber. As seen in Figure 7, the C/EBPbeta-1 expressing MDA-231 and MDA-435 cells displayed a substantial reduction in invasion approaching 80%. Invasive activity was normalized to that of uninfected breast cancer cells included in each assay, This reduction was not observed in the control GFP-expressing cells. **We conclude that C/EBPbeta-1 expression is a potent inhibitor of invasive growth by mammary epithelial cells in culture.**

We extended these studies using the nude mouse xenograft model as outlined in the Statement of Work. HisC/EBPbeta-1-IRES-GFP infected MDA 231 cells were first sorted by FACS to remove any uninfected or low expressing cells (see C/EBPbeta-3 below) and then implanted into the mammary pad of 10 female nude mice. We also implanted control GFP-infected and sorted cells into a separate group of 10 nude mice. However, in addition to monitoring tumor latency and size as endpoints, we also compared metastatic potential of the implanted cells. MDA 231 cells from the primary tumor typically metastasize to lungs of nude mice in 10-12 weeks (or by the time the primary tumor has reached approximately 1 cm) with nearly 100% frequency. We expected that C/EBPbeta-1 expressing MDA 231 cells would not metastasize or do so with lower frequency or reduced number of lung colonies. Both the MDA 231 cells and C/EBPbeta-1-MDA 231 cells formed tumors with similar latency. Tumor size ranged from 0.5cm to 2.5cm although there was no relationship between smaller tumor size and C/EBPbeta-1 expression. However, upon sacrifice, no lung metastases were found from either cells.

We do not know why the MDA 231 cells (which were obtained from Dr. Carlos Arteaga at Vanderbilt) did not metastasize as is usually seen. However, we obtained a new culture of these cells from the American Type Tissue Culture collection. The new MDA 231 cells were infected with hisC/EBPbeta-1-IRES-GFP retrovirus or IRES-GFP retrovirus were sorted by FACS. To ensure that the new MDA 231 cells responded similarly to C/EBPbeta-1, matrigel growth assays were performed and results indeed matched those of Figure 5,6. Unfortunately, during this time the GFP-MDA231 control cells became contaminated. We had to reinfect with IRES-GFP retrovirus and resort this population. Two important changes were made to the xenograft assay. First, we chose to use SCID/beige rather than nude mice. This is because our colleagues in the Arteaga lab, who have observed lung metastases with MDA231 cells , have seen

decreased latency and increased number of metastasis in the SCID/beige mice. Second, to avoid any variability in primary tumor growth which might in turn affect metastasis, we performed direct experimental metastasis assays. In this approach, tumor cells are injected directly into the circulation of immunocompromised mice via the tail vein or retroorbital injection (5). This also bypasses local invasion and intravasation. Evidence is accumulating that these early steps in the metastatic cascade, may in fact not be rate limiting (6). For these experiments, 1 x 105 cells, either MDA231-C/EBPbeta-1+GFP or MDA231+GFP were retroorbitally injected into 4-6wk old female SCID beige mice. Parental MDA 231 cells were injected as a positive control. All injections were completed between May 20-28. After 4 weeks, or if mice become moribund sooner, they will be sacrificed and numbers of lung metastases determined by visual inspection. Samples of lung tissue will also be processed in the mouse pathology core for immunofluorescent staining to visualize taggedC/EBPbeta-2 (and also GFP) in the tumor cells. We expect that animals receiving C/EBPbeta-2 expressing cells will exhibit a substantial increase in lung metastases when compared to C/EBPbeta-1+GFP, GFP only, or parental controls. In all cases differences between treatment groups will be analyzed by one-way analysis of variance (anova) for statistical significance.

C/EBPbeta-3

In year 01, we infected the breast cancer cell line, MDA 231, with hisC/EBPbeta-3-IRES-GFP retrovirus. hisC/EBPbeta-3 expression in the cells was monitored by immunofluorescent staining with T7tag antibody and shown to be entirely nuclear, as expected (Figure 8). Immunoblotting also confirmed substantial overexpression of C/EBPbeta-3 in the infected cells, clearly equaling or exceeding the amount of C/EBPbeta-2 endogenously present in the cells (Figure 9). We demonstrated that MDA 231 cells overexpressing C/EBPbeta-3 are growth inhibited and drop from 50% to 8% of the population over a 2 week interval (Figure 10). In contrast, the number of GFP positive cells in the control IRES-GFP infected population is stable over the 4 week time course of the experiment, indicating that GFP alone does not impair the growth of the cells. The data in Table 1 from cell cycle profiles on the GFP positive cells overexpressing C/EBPbeta-3 at day 17 postinfection suggest that this isoform of C/EBPbeta may cause growth arrest through a G2/M block .

TABLE ONE: Cell Cycle Distribution of MDA 231 cells expressing C/EBPbeta-3.

Cell Line	Go/G1	S	G2/M
MDA 231-C/EBPbeta-3/GFP	38%	39%	23%
MDA 231-GFP	47%	40%	13%

MDA 231 cells at 17 days postinfection with LZRShisC/EBPbeta-3-IRES-GFP or LZRS/IRES/GFP retrovirus were stained with propidium iodide and sorted by FACS. The diploid model was used to analyze cell cycle profiles.

Given these encouraging results, in year 02 we determined whether growth inhibition in culture extended to tumor formation in vivo in nude mice as outlined in the Statement of Work for the period 12-24 months. Xenograft studies, in which C/EBPbeta-3 expressing MDA 231 cells were implanted into the mammary pad of 10 female nude mice, showed no reduction in tumor size compared with control uninfected, or GFP expressing cells implanted in populations of 10 nude mice each. However, the latency of tumor formation was delayed. We attribute this outcome to the presence of cells in the population expressing a low level of C/EBPbeta-3 which is not sufficient to inhibit growth. To avoid this problem, we would need to sort hisC/EBPbeta-3-IRES-GFP infected MDA 231 cells by FACS, setting the gate to select only GFP positive cells of high fluorescent intensity. Since hisC/EBPbeta-3 and GFP are translated from the same bicistronic mRNA, those cells expressing high levels of GFP are also expressing high levels of hisC/EBPbeta-3. Therefore, we would expect to remove the cells expressing only a low level of hisC/EBPbeta-3 prior to implantation. However, we did not implement this approach due to lack of funds. The salary of the postdoctoral fellow on this grant, Dr. Bundy, increased from the 36,036 budgeted in year 01 to 42,000 in year 02 and 45,000 in year 03. This was required to maintain alignment with increases in the recommended level of support for postdoctoral fellows instituted by the NIH, and is far in excess of the 3% annual increase budgeted (which would lead to salaries of 37,117 and 38,230 in years 02 and 03 respectively). Thus, Dr. Bundy's salary has exceeded budget by $11,650 in years 02 and 3 of the grant combined. Consequently, because not all xenograft studies could be repeated, we chose to pursue the direct experimental metastasis assay with C/EBPbeta-1-expressing MDA 231 cells as described above, because of the potential for identifying a new inhibitor of metastasis.

Task 5. **To infect a representative panel of breast cancer cell lines with both C/EBPβ-3 and C/EBPβ-1 and analyze growth potential of the infected cells (months 18-36).**

To pursue these studies we used MDA-231 cells expressing C/EBPbeta-1 (no GFP) and infected them with Ad-C/EBPbeta-3. Since our Ad-C/EBPbeta-3 virus also expresses GFP, we also infected MDA 231 cells with Ad-GFP as a control. Much to our surprise, infection with Ad-GFP completely inhibits the growth of the MDA-231 cells expressing C/EBPbeta-1. This does not occur with the parental MDA 231 cells. We do not think that the expression of GFP is toxic to MDA 231 cells expressing C/EBPbeta-1 as we have introduced both of these proteins together in MDA231 cells via our LZRShisC/EBPbeta-1/IRES/GFP retrovirus many times (see Figures 1, 2, 3, and 5). Rather we think that MDA-231 cells expressing C/EBPbeta-1 are unusually sensitive to adenovirus infection and it is this process or some adenoviral protein expressed during this process that is toxic to the cells. Therefore, we will need to develop some means other than adenoviral infection to express C/EBPbeta-3 in the C/EBPbeta-1 expressing MDA 231 cells. One possibility is to construct a retrovirus that encodes both C/EBPbeta-1 and C/EBPbeta-3 as separate proteins from a single mRNA via an IRES, much like we have done for C/EBPbeta-1 or –3 alone with GFP. However, because we have come to the end of our time frame for support, that project will have to await funding via some future award.

KEY RESEARCH ACCOMPLISHMENTS

4. Development of high titer, non-replicating retrovirus to selectively express high levels of C/EBPbeta-1 in mammalian cells. Because the virus is amphotropic, both human and murine cells can be infected. GFP expression is included as a selectable marker, allowing infected cells to be easily quantified and/or sorted.

5. Developed high titer, non-replicating retroviirus to selectively express high levels of C/EBPbeta-3 in mammalian cells. Because the virus is amphotropic, both human and murine cells can be infected.

GFP expression is included as a selectable marker, allowing infected cells to be easily quantified and/or sorted.

6. Developed high titer, non-replicating adenovirus to selectively express high levels of C/EBPbeta-3 in mammalian cells.

7. Overexpression of C/EBPbeta-3 potently inhibits the growth of MDA 231 breast cancer cells in culture.

8. Reexpression of C/EBPbeta-1 blocks the invasive growth of human breast cancer cells in culture.

REPORTABLE OUTCOMES

Manuscripts:

Eaton, E., Hanlon, M., Bundy, L., and Sealy, L. (2001) Characterization of C/EBPbeta Isoforms in Normal vs. Neoplastic Mammary Epithelial Cells. *J. Cell. Physiology* **189**, 91-105.

Eaton, E. and Sealy, L. (2003) Reexpression of C/EBPbeta-1 in breast cancer cells blocks invasive growth. *Oncogene,* in preparation.

Sealy, L. (2003) Expression of C/EBPbeta-3 (LIP) inhibits breast cancer cell growth in culture. *BMC Cell Biology,* in preparation.

Abstracts:

Bundy, L.M., Eaton, E., Hanlon, M., and Sealy, L. (2001) Regulation of Mammary Epithelial Cell Growth by CCAAT/Enhancer Binding Protein Beta. *Mammary Gland Gordon Conference*, June 3-8, 2001.

Linda Sealy, Linda M. Bundy, Erin Eaton, and Mary Hanlon. (2002) Regulation of Mammary Epithelial Cell Growth by CCAAT/Enhancer Binding Protein (C/EBP) Beta. *DOD Era of Hope Meeting,* Sept. 2002.

Personnel receiving salary support from this grant:

Linda Sealy, Ph.D. – Principal Investigator

Linda Bundy, Ph.D. – Postdoctoral fellow

CONCLUSIONS

In this first year of this grant we successfully developed several powerful tools to directly test whether C/EBPbeta-1 and –3 isoforms, which are missing or expressed at a low level in breast tumor cells, could be an effective anti-tumor therapy. We have in hand high titer, non-replicating retroviruses that encode either C/EBPbeta-1 or –3 and a high titer, non-replicating adenovirus that encodes C/EBPbeta-3. These viruses are capable of efficiently infecting human cells, so that after a single infection between 70 and 90% of the cells express the C/EBPbeta isoform of interest . The addition of an IRES and GFP to the retroviral constructs was a valuable modification, in that not only are the infected cells quickly identified, but they can also be sorted via FACS to provide a uniform population for study, without the need for lengthy drug selection. This is essential for the analysis of growth inhibitory proteins.

In the second and third years of the grant period, we analyzed the growth potential of breast cancer cell lines infected with C/EBPbeta-1 or –3 viruses. We found that although C/EBPbeta-1 may have some effects on restoring contact inhibition, clearly the most dramatic result is the complete inhibition of invasive growth in culture. Xenograft studies, currently ongoing, also appear promising in reducing or blocking metastatic growth of tumor cells by C/EBPbeta-1. C/EBPbeta-2 is also a potent inhibitor of breast cancer cell growth in culture. Our initial xenograft studies of C/EBPbeta-3 infected breast cancer cells were not successful. However, we can address the problem of low expressing cells in the population using FACS sorting, and would like to repeat these studies in the future. We remain optimistic that combined expression of both C/EBPbeta-1 and –3 (task 5, year 3) will prove to be the most potent anti-tumor therapy in xenograft animal models, since both primary tumor growth (via beta-3) and metastatic potential (via beta-1) will be targeted. The problem we encountered with adenovirus infection of C/EBPbeta-1 expressing cells could not have been foreseen. However, the future development of a bicistronic retroviral vector encoding both C/EBPbeta-1 and –3 is likely to be a valuable anti-tumor tool.

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APPENDIX

Contents:

Figures 1-10

Abstract: Bundy, L.M., Eaton, E., Hanlon, M., and Sealy, L. (2001) Regulation of Mammary Epithelial Cell Growth by CCAAT/Enhancer Binding Protein Beta. *Mammary Gland Gordon Conference*, June 3-8.

Abstract: Linda Sealy, Linda M. Bundy, Erin Eaton, and Mary Hanlon. (2002) Regulation of Mammary Epithelial Cell Growth by CCAAT/Enhancer Binding Protein (C/EBP) Beta. *DOD Era of Hope Meeting,* Sept. 2002.

Reprint: Eaton, E., Hanlon, M., Bundy, L., and Sealy, L. (2001) Characterization of C/EBPbeta Isoforms in Normal vs. Neoplastic Mammary Epithelial Cells. *J. Cell* ***Physiology*** 189, 91-105.

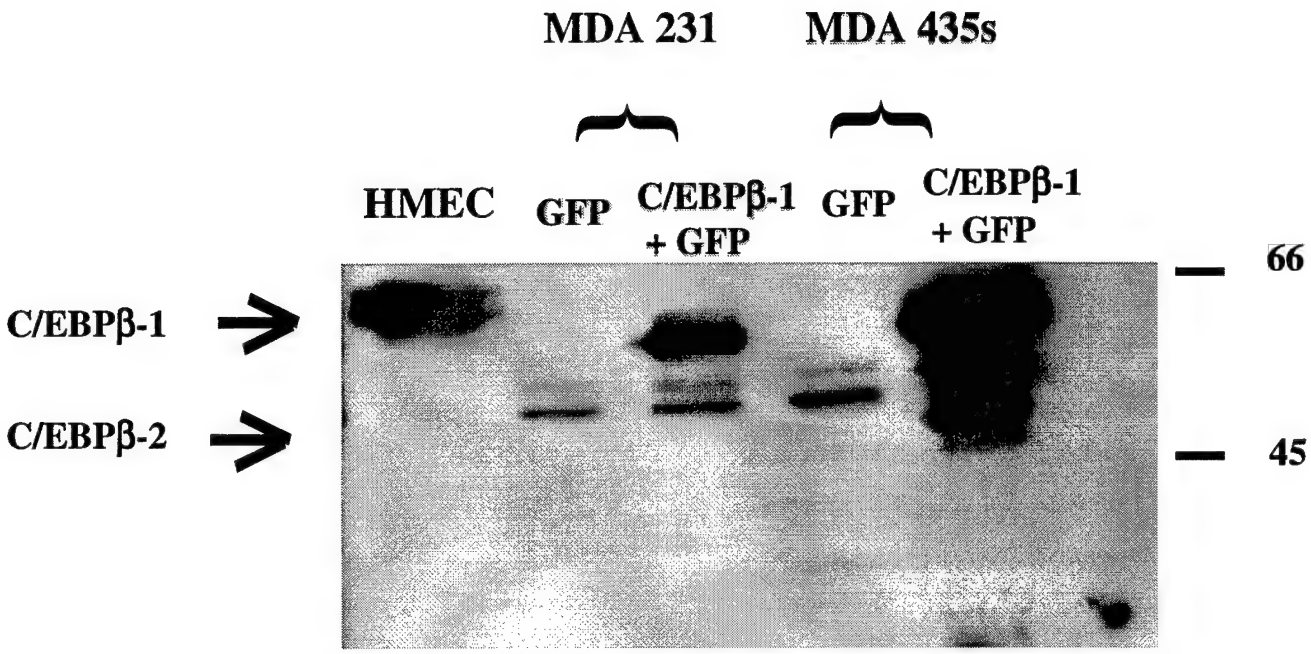


Figure. 1. Retrovirally transduced C/EBPbeta-1 is expressed in breast cancer cell lines at levels similar to normal HMECs.

Nuclear extracts from MDA231 or MDA 435 cells infected with either C/EBPb-1 and GFP (left) or GFP alone (right) were subjected to immunoblot analysis using the C-terminal C/EBPb antibody from Santa Cruz.

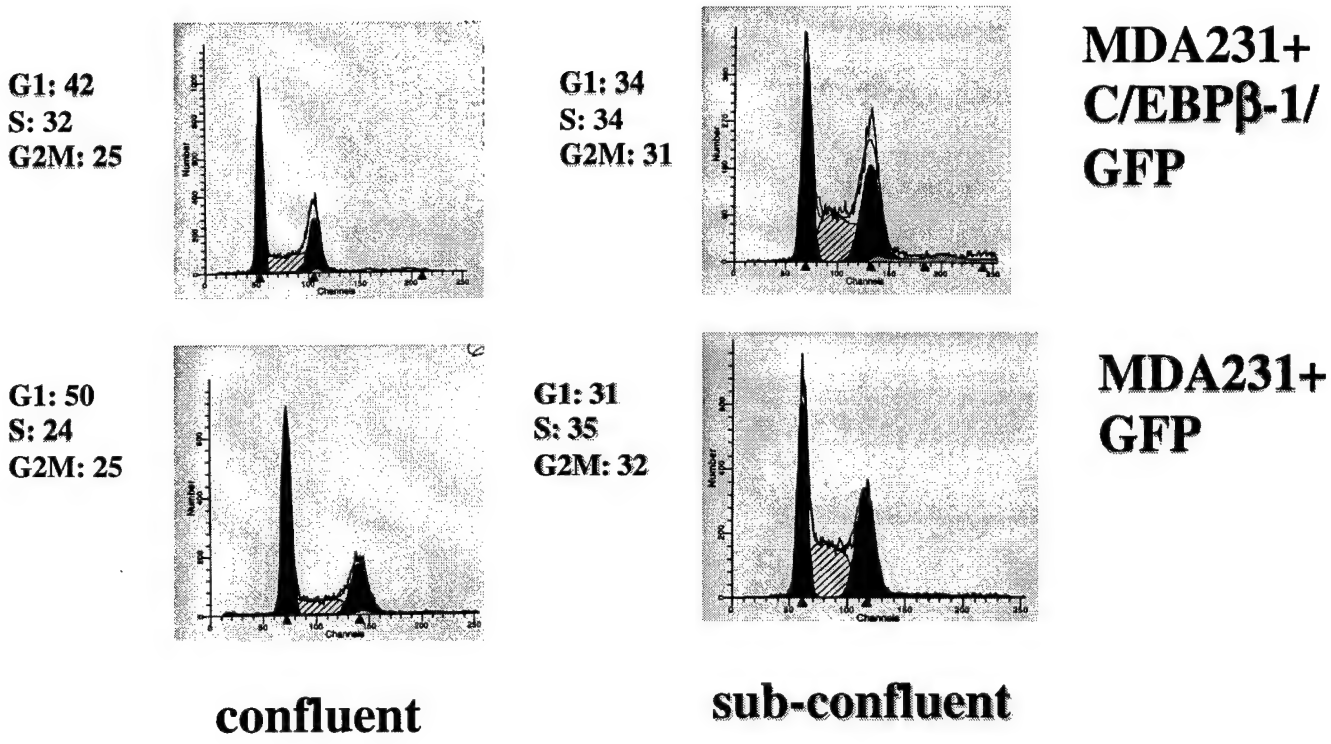


Figure 2. Cell cycle profile of MDA231 cells expressing C/EBPbeta-1 or GFP.

Subconfluent or confluent cultures of MDA231 cells expressing C/EBPbeta-1 and GFP or GFP only were stained with propidium iodide and cell cycle profiles acquired by FACS.

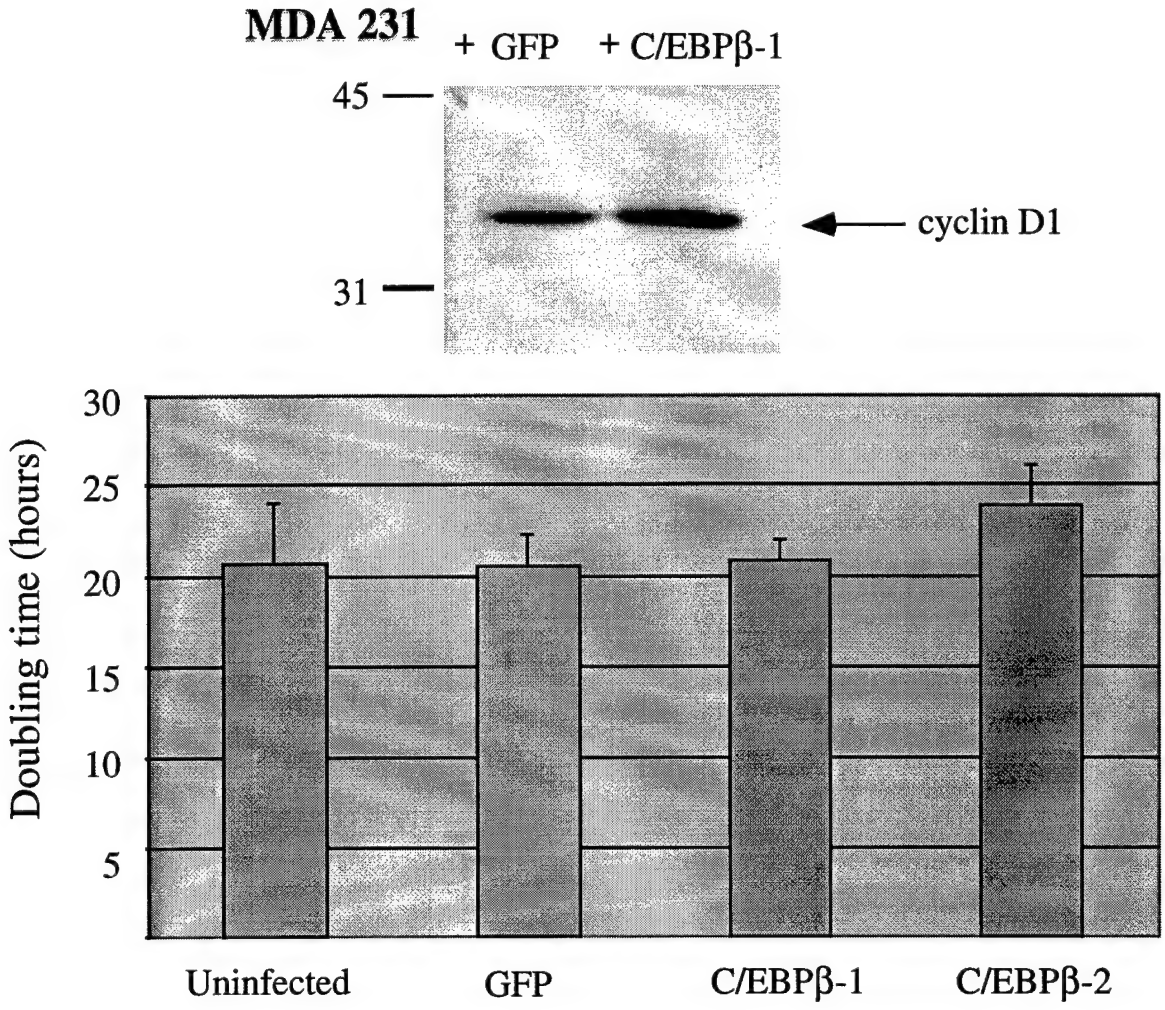


Figure 3. Doubling times and cyclin D1 levels ar unaffected by C/EBPbeta-1 expression.

Doubling times of MDA231 cells either uninfected, GFP infected, C/EBPbeta-1 or C/EBPbeta-2 infected, were determined by plating 1 X 10^5 cells in 100mm dishes. After 48 hrs cells were harvested and counted. After counting, cells were replated at the above density and allowed to grow for another 48 hrs before another count was taken. This was repeated for a total of 8 data points. Error bars represent sstandard error of the data. Cyclin D1 levels were determined in whole cell extracts of MDA231 cells expressing C/EBPbeta-1/GFP or GFP only by immunoblotting with human anti-cyclin D1 antibody from Pharmingen.

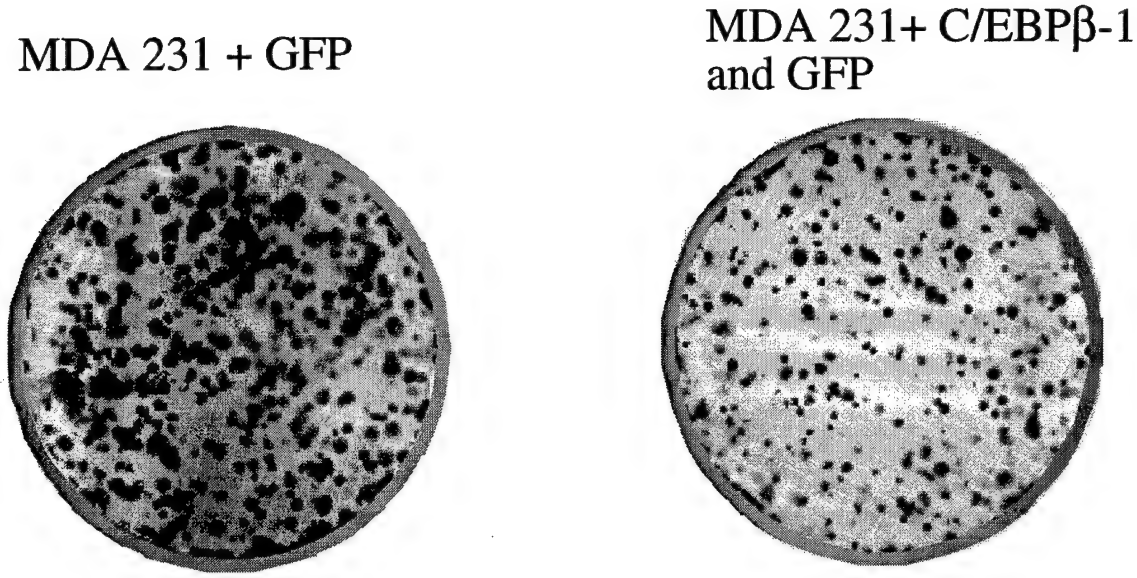


Figure 4. MDA 231 breast cancer cells expressing C/EBPbeta-1 form smaller colonies in a growth assay.

800 cells were plated and then allowed to grow for 8 days. Shown are representative plates stained with hematoxylin.

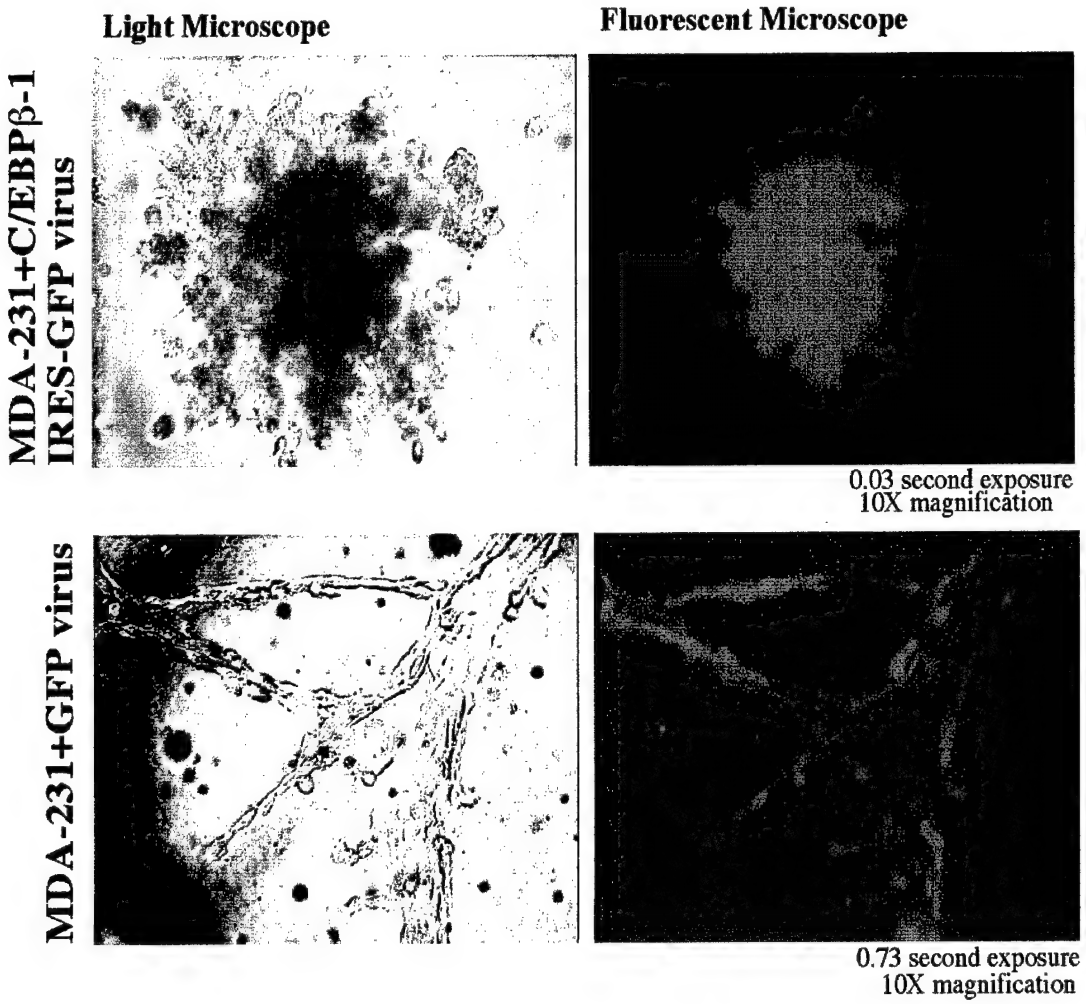


Figure 5. Expression of C/EBPbeta-1 completely blocks the branching growth of MDA231 cells in 3D culture.

Matrigel outgrowth assays were performed as in () with MDA 231 cells expressing C/EBPβ-1 and GFP (top) or GFP only (bottom).

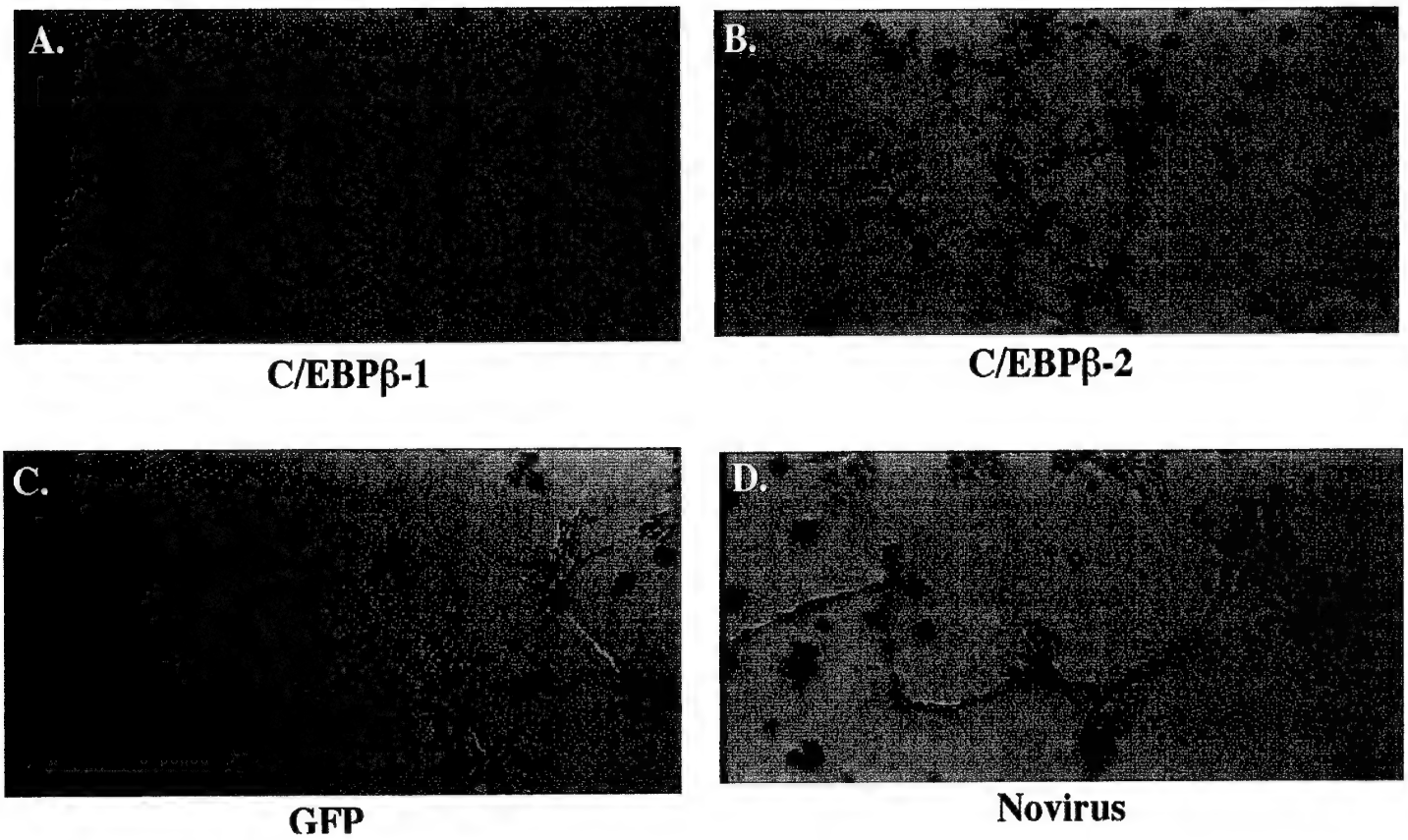


Figure 6. C/EBPbeta-1, but not C/EBPbeta-2 blocks the invasive growth of MDA 435 cells in 3D culture.

Growth morphology in matrigel of MDA 435 cells expressing C/EBPβ-1 and GFP (A); C/EBPβ-2 and GFP (B) GFP only (C) or no treatment (D). Cells in A-C were selected for green fluorescence by FACS prior to placement in matrigel.

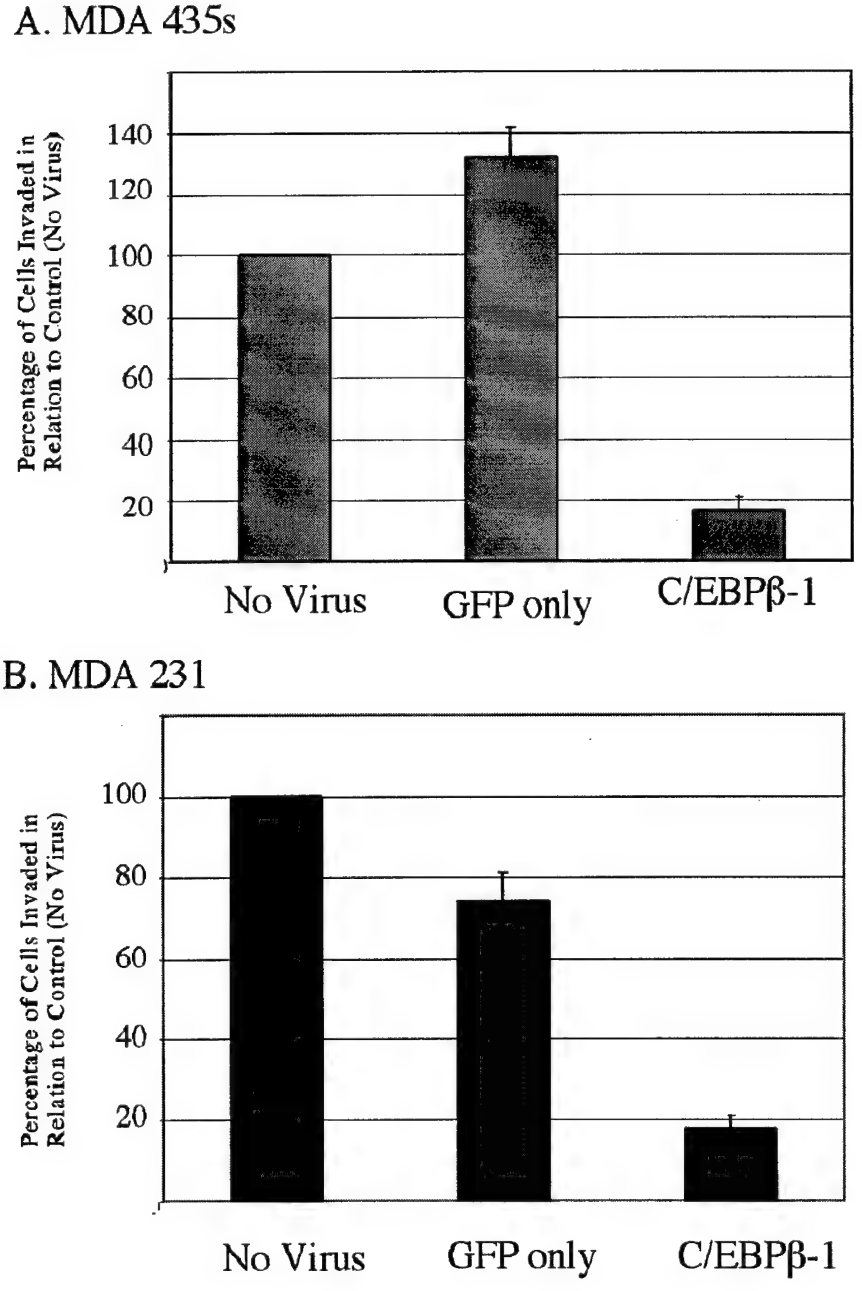
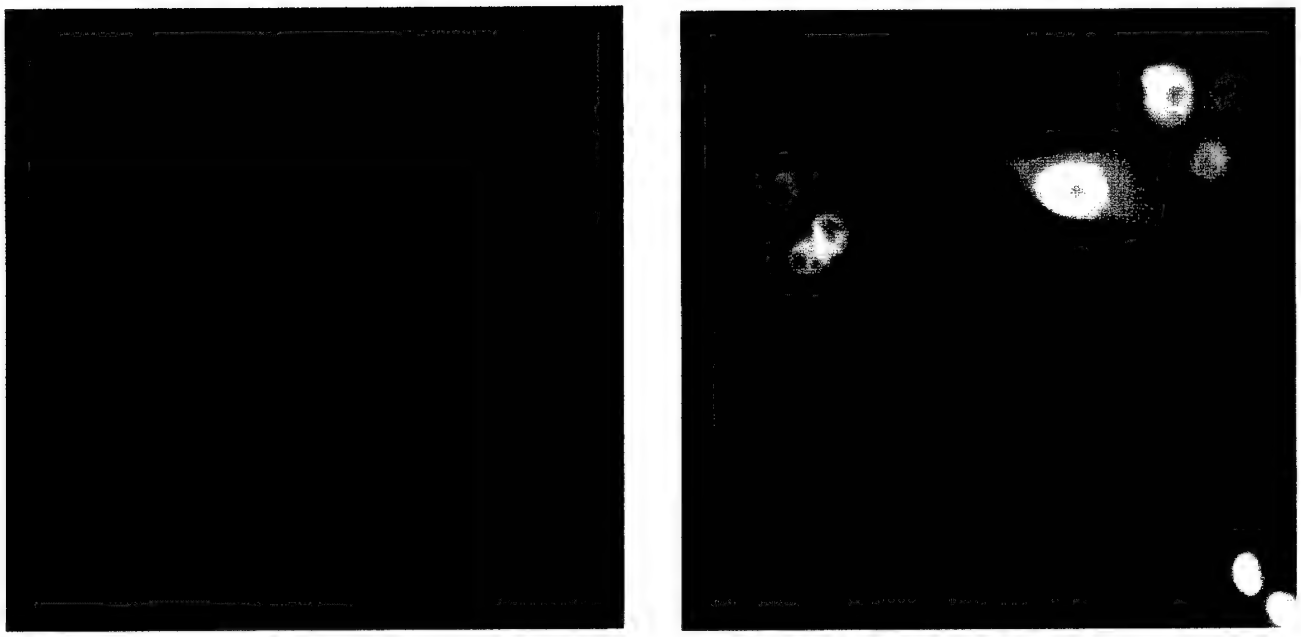


Figure 7. Introduction of C/EBPbeta-1 into breast cancer cell lines inhibits their invasive potential in vitro.

Uninfected cells, cells expressing only GFP, or cells expressing GFP and C/EBPβ-1 (MDA 231-black bars, MDA 435-gray bars) were placed in modified Boyden chambers. After 18 hrs, cells on the underside of the filter were stained and counted. Invasion is expressed as % of control (uninfected cells). Data shown are results from 3 independent experiments performed in duplicate.



T7-tag Ab GFP+T7-tag Ab

Figure 8. hisC/EBPbeta-3 expression in MDA 231 cells.

Immunofluroescent microscopy of LZRShisC/EBPbeta-3/IRES/GFP infected MDA 231 cells stained with primary anti-T7 epitope and secondary Alexa 594 antibodies (red, left panel) or overlaid with green GFP immunofluorescence (right panel) demonstrating nuclear expression of hisC/EBPbeta-3.

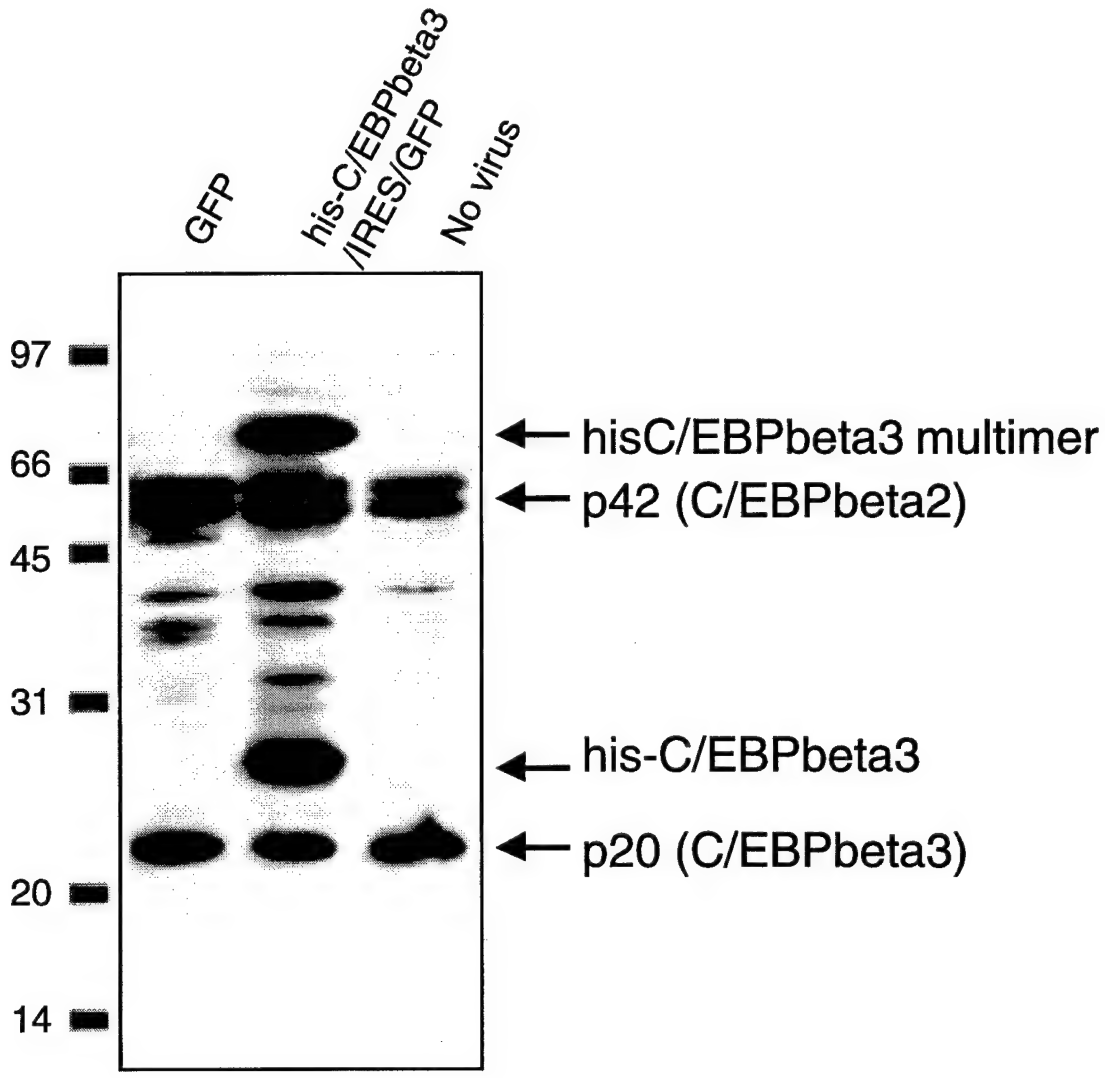


Figure 9. Expression level of hisC/EBPbeta-3 in LZRShisC/EBPbeta-3/IRES/GFP infected MDA 231 cells.

Whole cell extracts were prepared from MDA 231 cells (no virus) or MDA 231 cells infected with LZRShisC/EBPbeta-3/IRES/GFP or LZRS/IRES/GFP retroviruses, analyzed by SDS gel electrophoresis and immunoblotted with anti-C/EBPbeta antibody.

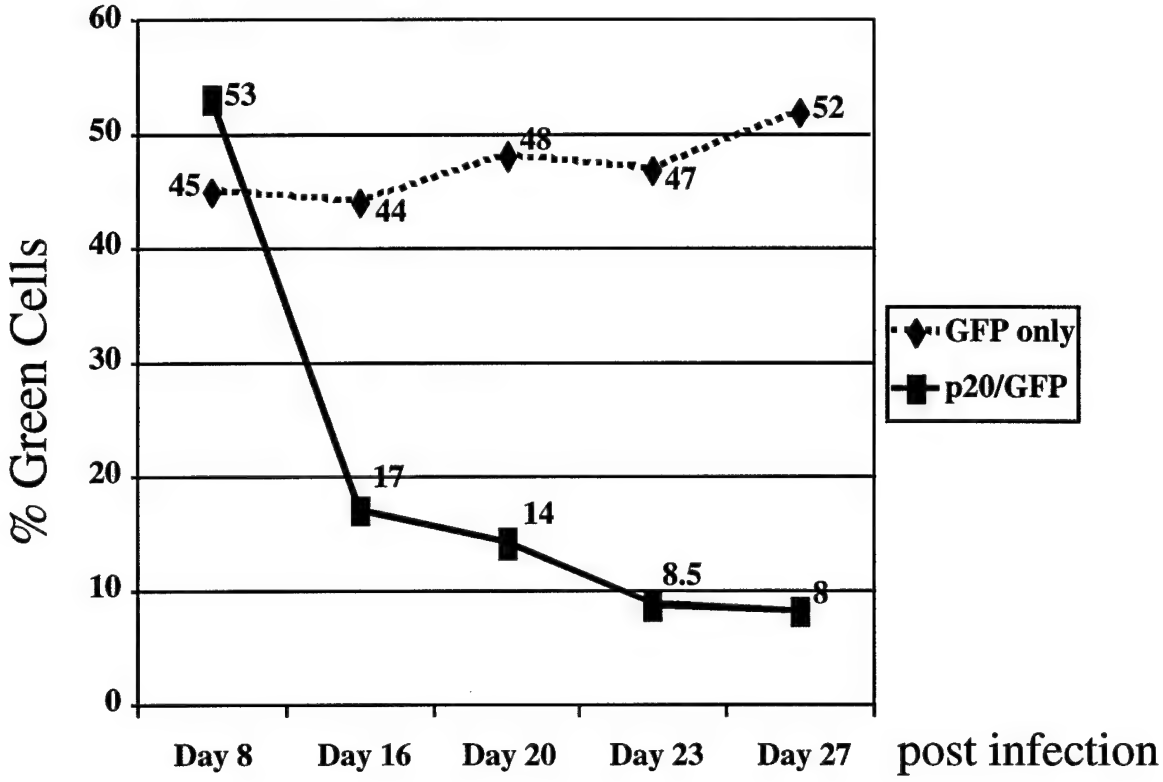


Figure 10. C/EBPbeta-3 inhibits the growth of MDA231 cells.

FACS analysis of LZRShisC/EBPbeta-3/IRES/GFP infected (p20/GFP) or LZRS/IRES/GFP infected (GFP only) MDA 231 cells to determine the percentage of GFP positive cells at the indicated day postinfection.

Regulation of Mammary Epithelial Cell Growth by CCAAT/Enhancer Binding Protein β

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ABSTRACT

Gene knockout studies show that the transcription factor, C/EBPβ, is critical for growth and differentiation of the mammary gland during puberty, pregnancy, lactation, and involution. Three forms of C/EBPβ can be expressed in cells. C/EBPβ-1 and C/EBPβ-2 activate transcription; C/EBPβ-3 is a truncated form that represses transcription. To examine the distinct functions of these isoforms, we have generated recombinant amphotropic retrovirus carrying genes selectively encoding epitope-tagged C/EBPβ of each isoform using a hybrid Epstein-Barr virus (EBV)/retroviral vector construct (LZRSpBMN-Z) developed and provided to us by G. Nolan (Stanford University).

Although C/EBPβ-1 and -2 are both transactivators, our laboratory has shown that they are differentially expressed. Using an antibody we developed that is specific for C/EBPβ-1, we made the novel discovery that C/EBPβ-1 is expressed in normal human mammary epithelial cells (HMECs) and ductal secretory epithelial cells harvested from human breast milk, but absent in all breast cancer cell lines examined. Moreover, MCF10As, an immortal but "normal" human mammary epithelial cell line, engineered to overexpress C/EBPβ-2 acquire transformed characteristics: they form foci, lose anchorage independence, become invasive, and express mesenchymal markers. Based upon these observations, and other data, we propose that C/EBPβ-1 promotes differentiation while C/EBPβ-2 promotes proliferation in mammary epithelial cells. We hypothesize that mammary tumors exhibit deregulated growth because of constitutive activation of C/EBPβ-2 in the nucleus, coupled in many cases with a lack of C/EBPβ-3. Lastly, we will present data demonstrating that overexpression of the dominant negative C/EBPβ-3 isoform acts as a potent inhibitor of growth in human breast cancer cell lines.

REGULATION OF MAMMARY EPITHELIAL CELL GROWTH BY CCAAT/ENHANCER BINDING PROTEIN (C/EBP) BETA

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Gene knockout studies show that the transcription factor, C/EBPbeta, is critical for growth and differentiation of the mammary gland during puberty, pregnancy, lactation, and involution. Three forms of C/EBPbeta can be expressed in cells by alternative translation initiation. C/EBPbeta-1 and C/EBPbeta-2 activate transcription; C/EBPbeta-3 is a truncated form that represses transcription. We have observed a striking difference in the expression profiles of C/EBPbeta-1 and –2 in normal and neoplastic human mammary epithelial cells (MECs) derived from both primary tissues and established cell lines. Specifically, C/EBPbeta-1 is expressed in normal human mammary epithelial cells (HMECs) from reduction mammoplasties and ductal secretory epithelial cells harvested from human breast milk, but is absent in all breast cancer cell lines examined, which express only the C/EBPbeta-2 transactivator. Normal breast tissue from reduction mammoplasties expressed exclusively C/EBPbeta-1, whereas 7 of 10 surgical primary breast tumor samples acquired elevated C/EBPbeta-2 levels.

To further study whether the two C/EBPbeta activator isoforms carry out distinct roles in MECs, we have exploited recombinant retroviral technology to selectively overexpress either epitope-tagged C/EBPbeta-2 or C/EBPbeta-1 in various human mammary cell lines. C/EBPbeta retroviruses with or without a marker, green fluorescent protein, were generated using a hybrid Epstein-Barr virus (EBV)/retroviral vector construct (LZRSpBMN-Z) developed and provided to us by G. Nolan (Stanford University). We found that elevated levels of tagged C/EBPbeta-2 resulted in dramatic transformation of a normal human mammary epithelial cell line, MCFA. MCF10A cells engineered to overexpress C/EBPβ-2 form foci, gain anchorage independence, express markers associated with having undergone an epithelial to mesenchymal transition (EMT), and acquire an invasive phenotype. Interestingly, when C/EBPβ-1 was overexpressed in the highly invasive MDA 231 cells, the invasive behavior in culture of these cells was completely reversed. C/EBPβ-1 is not expressed in MDA 231 cells; reintroducing this isoform led to the adoption of a fused/spherical morphology in Matrigel, which is typical of less invasive breast cancer cell lines. Based upon these observations, and other data, we propose that C/EBPβ-2 activates genes which promote invasive cell growth, whereas the biological role of C/EBPβ-1 is to activate differentiation specific gene transcription . These studies provide supportive evidence that deregulated expression of C/EBPβ-2 contributes to malignant conversion of the human breast.

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Characterization of C/EBPβ Isoforms in Normal Versus Neoplastic Mammary Epithelial Cells

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A member of the CCAAT Enhancer Binding Proteins (C/EBPs) family of transcription factors, C/EBPβ, has recently proven to be an important player in both growth and differentiation of the epithelial cells in the mammary gland. When the gene for C/EBPβ is disrupted in mice, these mice fail to either develop normal mammary ducts during puberty or pregnancy, or to lactate upon parturition. C/EBPβ can be present in cells in three isoforms: C/EBPβ-1, -2, and -3. These isoforms have the same carboxy terminus but different N-termini due to alternative translational initiation at three different initiator codons within the C/EBPβ mRNA. Using a commercially available antibody specific to the C-terminus of C/EBPβ and a novel antibody specific to the N-terminus of C/EBPβ-1, we have uncovered a striking difference in the forms of C/EBPβ present in normal mammary epithelial cells versus breast cancer cell lines. C/EBPβ- 1 is found exclusively in normal mammary epithelial cells, whereas C/EBPβ- 2 is found only in dividing cells, both normal and neoplastic. Our preliminary data suggest that the prevalent form of C/EBPβ in cancer cells, C/EBPβ- 2, can activate genes which push the cell to divide, such as cyclin D1. J. Cell. Physiol. 189: 91–105, 2001. © 2001 Wiley-Liss, Inc.

The CCAAT Enhancer Binding Proteins (C/EBPs) are a family of basic leucine zipper transcription factors. These proteins play a variety of roles in multiple different cell types (reviewed in Lekstrom-Himes and Xanthopoulos, 1998). One member of this family, C/EBPβ, has recently proven to be essential in mammary epithelial cells. Transgenic mice, which lack the C/EBPβ gene, fail to develop normal mammary glands at puberty or pregnancy, or to lactate upon parturition (Robinson et al., 1998; Seagroves et al., 1998). These mice, therefore, display a dual phenotype: a defect in mammary epithelial cell proliferation in response to hormonal stimulation at puberty or pregnancy and a defect in mammary epithelial cell differentiation in response to lactation specific hormones. Failure of these mice to lactate is perhaps not too surprising, since C/EBPβ has been implicated in the regulation of milk-specific genes such as beta-casein (Doppler et al., 1995; Raught et al., 1995). Failure of the epithelial cells to proliferate at puberty or pregnancy is more unexpected, as C/EBPβ is commonly thought regulate differentiation specific genes rather than proliferation specific genes.

Alternative translation of the intronless C/EBPβ gene produces three different protein isoforms possibly due to leaky ribosome scanning (Descombes and Schibler, 1991). However, an alternative mechanism recently has been proposed involving regulation by the mRNA binding protein CUGBP1 (Timchenko et al., 1999). C/EBPβ-1 and -2, initiated at the first and second in-frame methionines respectively, activate transcription. Either translation initiation at the third in-frame methionine, or, as recent studies suggest, proteolytic cleavage of C/EBPβ (Baer and Johnson, 2000), generates a small 20 kD protein, C/EBPβ- 3, which lacks the N-terminal transactivation domain and therefore represses transcription (see Fig. 1). Only recently have groups begun to study the functional differences between the two activator proteins (Kowenz-Leutz and Leutz, 1999). Because human C/EBPβ-1 and C/EBPβ-2 differ by only 23 amino acids (the mouse, rat, and chicken proteins differ by only 21 amino acids), it is difficult to distinguish between phosphorylated C/EBPβ-2 and C/EBPβ-1 using the commercially available antibody, which recognizes an epitope at the C-terminus of the protein. To unequivocally distinguish the activator isoforms, we developed an antibody specific to the N-terminal amino acids present in human C/EBPβ-1 but absent in C/EBPβ-2. Using this antibody, we have observed a striking difference in the expression of

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these two activator isoforms between human breast cancers and normal human breast cells from both established human cell lines and primary tissues. C/EBPβ-1 is present in all normal cells and tissues examined but absent in all the breast cancer cell lines examined. C/EBPβ-2 is found only in actively or potentially dividing cells but not in mainly quiescent differentiated tissues.

To determine whether the distinct expression profiles could also have functional significance, we examined a gene known to be important in breast development and carcinogenesis, cyclin D1. Cyclin D1 regulates the G1-S phase of the cell cycle. It and its catalytic partners, the cyclin dependent kinases(CDKs) 4 and 6, phosphorylate pRb. This phosphorylation inactivates pRb, which leads to the release of the transcription factor E2F, a protein necessary to activate genes involved in DNA replication (reviewed in Sherr, 1996). Deletion of the cyclin D1 gene in transgenic mice creates a phenotype in the mammary gland very similar to that seen in the C/EBPβ knockout mice (Fantl et al., 1995; Sicinski et al., 1995) in that mammary glands of these mice fail to proliferate upon hormonal stimulation at puberty or pregnancy. Cyclin D1 is implicated in breast cancer as well. Transgenic mice overexpressing cyclin D1 in the mammary gland via the MMTV promoter develop mammary hyperplasia and carcinoma (Wang et al., 1994). The cyclin D1 gene is amplified in 15–20% of breast cancers; the protein or mRNA is overexpressed in approximately 50% of breast cancers (Buckley et al., 1993; Bartkova et al., 1994).

In transient transfections, C/EBPβ-2 activates the cyclin D1 promoter while C/EBPβ- 1 does not. In fact, it slightly represses this promoter. Taken together with their different expression profiles, we propose that the two C/EBPβ activator proteins have functionally distinct roles within mammary epithelial cells with C/EBPβ-2 promoting growth and C/EBPβ-1 regulating the transcription of genes in non-proliferating or differentiating cells.

MATERIALS AND METHODS

Western analyses

Whole cell extracts were prepared from subconfluent cultures of breast cancer cell lines and 81N cells (kind gifts of Dr. Carlos Arteaga, Vanderbilt University) or normal mammary epithelial cells in culture (HMECs from Clonetics, Walkersville, MD) or immortalized MCF10A cells (ATCC, Rockville, MD) by scraping the cells at 4°C into STE (10 mM Tris pH 8, 1 mM EDTA, 100 mM NaCl) plus protease/phosphatase inhibitors (10 μM Na vanadate, 10 mM Na molybdate, 10 mM beta-glycerolphosphate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride), adding an equal volume of 2 × Laemmli sample buffer (Biorad, Melville, NY) and boiling for 10 min. Mammary ductal epithelial cells (MDEs) were collected from freshly collected breast milk of one of the authors of this paper. Milk samples were centrifuged at 1,000*g* for 5 min. Supernatant was aspirated then cells were washed in phosphate buffered saline (PBS) plus 0.1 mM Na Vanadate at 4°C and recollected at 1,000*g* for 5 min. The cell pellets were resuspended in STE plus protease/phosphatase inhibitors and an equivalent volume of 2 × Laemmli sample buffer as above. Crude nuclear and cytoplasmic extracts were prepared from the cell lines by gentle lysis at 4°C in Buffer A (10 mM HEPES, pH 8, 0.5 M sucrose, 50 mM NaCl, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, and 7 mM β-mercaptoethanol) plus the protease/phosphatase inhibitors given above, followed by centrifugation at 600*g*. The supernatant (cytoplasm) was removed and combined with an equivalent amount of 2 × Laemmli sample buffer and the pellet (nuclei) was resuspended in 1 × Laemmli sample buffer. Relative protein concentrations were determined using Biorad Protein Assay Reagent, as described by the manufacturer, and equivalent amounts of sample were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblotting was performed as previously described (Hanlon and Sealy, 1999) using C/EBPβ antibody specific to the C terminus of C/EBPβ (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) or N terminal C/EBPβ antibody.

N-terminal antibody synthesis

A rabbit polyclonal antibody was raised to a KLH-conjugated 16 amino acid peptide (MQRLVAWD-PACLPLPP) corresponding to amino acids 1–16 of human C/EBPβ (also called NF-IL6). This 16 aa peptide

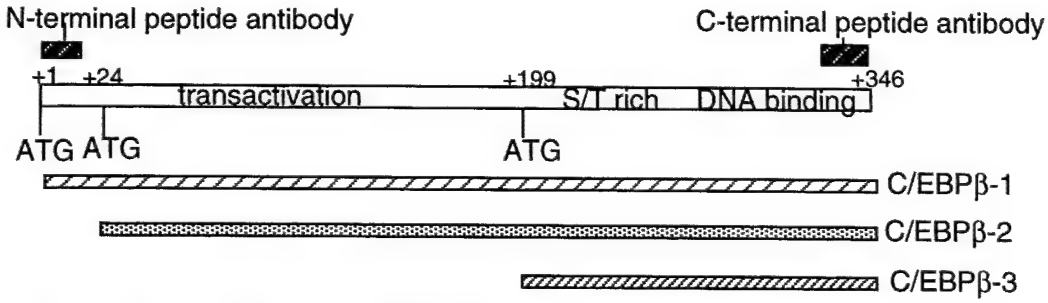


Fig. 1. Schematic representation of the C/EBPβ protein isoforms, showing ATG start sites of the three protein isoforms as well as binding sites for the antibodies used in the following experiments. Numbering is for the human C/EBPβ gene, NFIL6. The rat gene, called LAP, is 297 amino acids, and the difference between rat C/EBPβ-1 and -2 (also called LAP* and LAP) is only 21 amino acids. Because of 4 amino acid differences in the N-terminal epitope between NFIL6 and LAP, the antibody recognizes human C/EBPβ-1 but LAP* poorly if at all.

is only 75% conserved in the rat and mouse C/EBPβ proteins (also called LAP) and we have found experimentally that the antibody raised to the above peptide is fairly human-specific, recognizing rat/mouse C/EBPβ-1 very poorly if at all. The antibody was generated at East Acres Biologicals (Southbridge, MA) following their protocols. To obtain affinity-purified antibody, antiserum from production bleeds was incubated with purified, bacterially-expressed NF-IL6 protein that had been immobilized on Immobilon-P filter strips overnight at 4°C. The antigen-antibody containing strips were washed at room temperature 4 times in Tris-buffered saline plus 0.05% Tween 20 (TBS-T), once with TBS-T containing 1M NaCl, and then a final wash with TBS-T. Antibody was eluted from the antigen-containing strips by incubation in 0.2 M glycine, pH 2.2 containing 0.2% phenol red. Eluted antibody was immediately neutralized by the addition of sufficient 1.5 M tris pH 9 to bring the pH to 7.0 and BSA was added to a final concentration of 1 mg/ml.

Immunoprecipitations

HMECs were incubated with Dulbecco's modified Eagles medium (DMEM) lacking methionine for 45 min, then labeled with 1.6 mCi/ml of ^{35}S methionine (1,175Ci/mmol) (NEN) in met-free DMEM for 3.5 h at 37°C. All further steps were carried out at 4°C. Cells were harvested and lysed as described before (Hanlon and Sealy, 1999). After clarification, cell extracts were precleared for 90 min with unblocked protein A agarose beads (Gibco, Gaithersburg, MD). Cell extracts were then incubated overnight with BSA-blocked Protein A agarose beads (Calbiochem, San Diego, CA) either alone, or with affinity purified N terminal antibody, or with affinity purified N terminal antibody which had been pre-incubated with excess N terminal peptide to which the antibody was raised. Beads were collected and extensively washed with antibody lysis buffer plus protease/phosphatase inhibitors as given above. The beads were resuspended in 1 × Laemmli SDS sample buffer and boiled 5 min before analyzing samples by SDS 12% polyacrylamide gel electrophoresis. The gel was dried and exposed to film for 5 days.

Primary tissue analysis

Samples of frozen tissue were obtained from the Tissue Procurement Core at Vanderbilt University. The samples were ground at 4°C in 3 ml homogenization buffer (50 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 2 mM DTT) plus protease/ phosphatase inhibitors (100 μM Na vanadate, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) using a polytron. 1% Triton X-100 was added to the samples which were then left on ice for 5 min before a 10 min, 12,000*g* centrifugation. Supernatant was used for protein concentration determination or added to an equal volume of 2 × Laemmli sample buffer and boiled for 5 min before being analyzed by SDS 12% polyacrylamide gel electrophoresis. Samples were electrophoresed and transferred to Immobilon-P as described above. Blots were probed as above with either C-terminal antibody or N-terminal antibody.

Cloning of cyclin D1 promoter

Genomic DNA from MDA 231 cells was isolated by standard procedures for use as template. PCR was performed on 1 μg of the template using the following primers synthesized by Genosys Biotechnologies (5′ primer: GGAAGATCTCGCTCACGAATTCAGTCCC and 3′ primer: GGATCTAGAGCTCGGCTCTCGCTTCTGC). The 1.192 kb PCR fragment was gel purified and cloned into the PCR Blunt vector from Invitrogen as per manufacturer's instructions. A 1.294 kb fragment containing the cyclin D1 promoter was then cut out of PCR Blunt using *Mlu*I and *Eco*RV, gel purified, and subcloned into the pGL3 Basic luciferase reporter vector from Promega, previously digested with *Mlu*I and *Sma*I.

Cell culture and transfections

HMECs were obtained from Clonetics and maintained in mammary epithelial cell growth medium (MEGM) from that company as per instructions. MCF10A cells were obtained from the ATCC and cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 2.5 mM L-glutamine and supplemented with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% horse serum (Sigma, St. Louis, MO). NIH 3T3 fibroblasts, from the ATCC, were cultured in Dulbecco's modified Eagle's medium with 10% calf serum (Colorado Serum Company).

HMECs were transfected with 2 μg of the cyclin D1/luciferase construct (pGL3cycD) and 2 μg of empty vector (CMV4) or 2 μg of expression vector for either C/EBPβ- 1 or C/EBPβ- 2 using Geneporter liposome(Gene Therapy Systems, San Diego, CA), following manufacturer's instructions. Cells were exposed to the DNA/liposomes for 5 h before the medium was removed and replaced with complete growth medium. Cells were harvested after 36 h. Luciferase assays were carried out using luciferase assay reagent from Promega (Madison, WI), per manufacturer's instructions.

NIH 3T3 cells were transfected with NovaFector (VennNova, Pompano Beach, FL) as per manufacturer's instructions for 8 h, using the same constructs and amounts as above except when 100 ng of an RSV-LTR/luciferase construct was used as the reporter gene.

Expression vectors

CMV-LAP (C/EBPβ- 2) was a gift of U. Schibler (University of Geneva, Geneva, Switzerland). CMV4 was developed in the laboratory of David Russell and has been previously described (Andersson et al., 1989). The pcDNA3.1-C/EBPβ-1 was constructed by an oligonucleotide mutagenesis approach. First, a pRSETC-NFIL6 construct was generated by digestion of CMV-NFIL6 (a kind gift of S. Akira) with *Sal*I, incubation at 4°C with DNA Polymerase I to generate blunt ends and digestion by *Eco*RI to release a 1,045bp fragment. This fragment was inserted into a pRSETC vector (Invitrogen) that had been previously digested with *Hind*III incubated at 4°C with DNA Polymerase I and digested by *Eco*RI to generate pRSETC-NFIL6. The following oligos (synthesized by Sigma-Genosys, The Woodlands, TX) were used to replace the wild type sequence of pRSETC-NFIL6 (restriction enzyme sites are in lower

case): TOP STRAND: 5′ gatctGCAGCTGGTACCATGG**GC**TA**C**C<u>ATG</u>**G**AACGCCTGGTGGCCTGGGACCCAG C ATGTCTCCCCCTGCCGCCGCCGCCGCCTGCCTTTAAATCC*<u>GGA</u>*GAAGtgg3′BOTTOMSTRAND:5′ ccaCTTC*<u>TCC</u>*GGATTTAAAGGCAGGCGGCGGCGGCGGCAGGGGGAGACATGCTGGGTCCCAGGCCACCAGGCGTT**C**<u>CAT</u>GG**TAGC**CCATGGTACCAGCTGCa 3′. The oligos were annealed and inserted into a pRSETC-NFIL6 construct that had been previously digested with *Bgl*II and *Msc*I to release a 106 bp fragment. The resulting pRSETC-C/EBPβ-1 construct contains a consensus Kozak sequence surrounding the first ATG (underlined) and the second ATG is mutated to a glycine (underlined and italicized). The residues that differ from the wild type NFIL6 sequence are shown in bold. The pRSETC-C/EBPβ- 1 oligo mutant was then digested with *Hind*III, incubated at 4°C with DNA Polymerase I to generate blunt ends and digested with *Bam*HI to release a 1,082bp fragment. This fragment was inserted into *Bam*HI and *Eco*RV digested pcDNA3.1hisA to generate the pcDNA3.1-C/EBPβ- 1 construct.

Gel shifts and immobilon analysis

Nuclear and cytoplasmic extracts from HMECs were run on adjacent lanes of a SDS gel and transferred to Immobilon. One lane containing nuclear extracts was cut into 3-mm slices, while parallel lanes of nuclear and cytoplasmic extracts were processed for Western analysis. These 3-mm slices were placed into elution solution (50 mM Tris, pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 100 μg/ml BSA, 1% Triton X-100, 20% glycerol, 10 mM DTT) a 4°C to recover DNA binding activity. Protein samples from the Immobilon slices representing approximately 97–25 kD were used in electrophoretic mobility shift assays as previously described (Sealy et al., 1997) along with a ^{32}P labeled 26 bp oligonucleotide representing one of the C/EBPβ sites (at −221) in the human beta-casein promoter which has been previously identified by other groups (Doppler et al., 1995; Raught et al., 1995) or the following 27 bp oligonucleotide containing the −559 C/EBPβ consensus site (underlined) from the human cyclin D1 promoter: ^{567}TAAATTAGT<u>TCTTGCAAT</u>TTACACGTG^{-541}.

RESULTS

Normal mammary epithelial cells, but not breast cancer cell lines, express C/EBPβ-1.

C/EBPβ mRNA gives rise to three different protein products due to alternative use by the ribosome of three translation initiation codons. Two of these proteins are transcriptional activators, differing only by 23 N-terminal amino acids present in human C/EBPβ-1 but absent in C/EBPβ-2. The third protein product, C/EBPβ-3, lacks the N-terminal transactivation domain and is therefore a repressor of transcription (Fig. 1). In order to examine the expression of these different isoforms in human breast cancer cell lines as well as normal mammary cell lines, a series of Western analyses was performed using an antibody specific to the C-terminus of C/EBPβ. Also included in this analysis was a single normal cell population, 81N (Band and Sager, 1989). As shown in Figure 2A, all human breast cancer cell lines had significant levels of C/EBPβ protein with an estimated MW on our gel system of 45–50 kD. (On the actual film this band appeared to be a closely spaced doublet of 46 and 49 kD in several lanes). In contrast, the rat or mouse C/EBPβ protein (also called LAP) is often observed to be a 35–38 kD doublet. However, the larger size of the human C/EBPβ protein (also called NF-IL6) is expected. This is because the NF-IL6 protein is composed of 346 amino acids, 49 amino acids longer than the LAP protein which is only 297 amino acids in length. Some of the breast cancer cell lines analyzed in Figure 2A also contained variable levels of a 21 kD protein likely to be C/EBPβ-3. (The rat/mouse C/EBPβ- 3 protein is also called LIP. Human C/EBPβ- 3 and LIP have nearly identical MWs of approximately 20–21 kD, because the extra amino acids in the human C/EBPβ protein reside primarily in the N-terminal half not included in C/EBPβ-3 or LIP). While the normal 81N cells also showed expression of a 45 kD C/EBPβ protein, strikingly, only they displayed a more slowly migrating isoform of C/EBPβ ranging from 51–59 kD (MW of 55 kD at the band center). Because the antibody used in this analysis was specific to the C-terminus of C/EBPβ, and can therefore potentially recognize all three isoforms, one possibility is that the most slowly migrating 55kD band is human C/EBPβ-1 and the 45–50 kD proteins are human C/EBPβ-2. However, it is also possible that the bands are not different isoforms but variously modified forms of the same isoform. Before embarking on further characterization of the novel 55 kD C/EBPβ protein in the normal cells, we wished to determine if this protein was unique to the 81N cells or commonly found in other normal human mammary epithelial cells. Therefore, we purchased from Clonetics normal human mammary epithelial cells (HMECs) that had been placed in culture from an individual undergoing reduction mammoplasty. These cells are primary cells, which have undergone no crisis to survive for prolonged peroids in culture. In Figure 2B we analyzed a whole cell extract prepared from the HMECs along with several of the same tumor cell lines used in Figure 2A. Once again all cells expressed 45–50 kD C/EBPβ proteins and various levels of p21 or C/EBPβ-3. However, only the HMECs contained a more slowly migrating form of C/EBPβ at 55 kD. Although the 81N cells were no longer available to us to analyze on the very same immunoblot as the HMECs, based upon its MW the 55kD species in HMECs could likely be the same as the slowly migrating species observed in the 81N cells.

To determine if the 55kD protein observed in normal human mammary epithelial cells is a different isoform of C/EBPβ not expressed in the tumor cells, we developed an antibody in our laboratory which is specific to the N terminal amino acids present in human C/EBPβ-1 but absent in C/EBPβ-2. Because this antibody can recognize only C/EBPβ-1, it can be used to definitively distinguish C/EBPβ-1 from C/EBPβ-2. To characterize the specificity of the antibody, we first expressed bona fide human C/EBPβ-1 in a non-mammary, non-human cell line (mouse NIH 3T3 cells) by transient transfection of an expression construct for NF-IL6. The CMV-driven NF-IL6 expression construct was modified to ensure that only C/EBPβ-1 would be expressed by mutating the 2nd ATG codon to glycine and creating a perfect Kozak consensus around the first ATG. When affinity-purified N-terminal C/EBPβ antibody was used

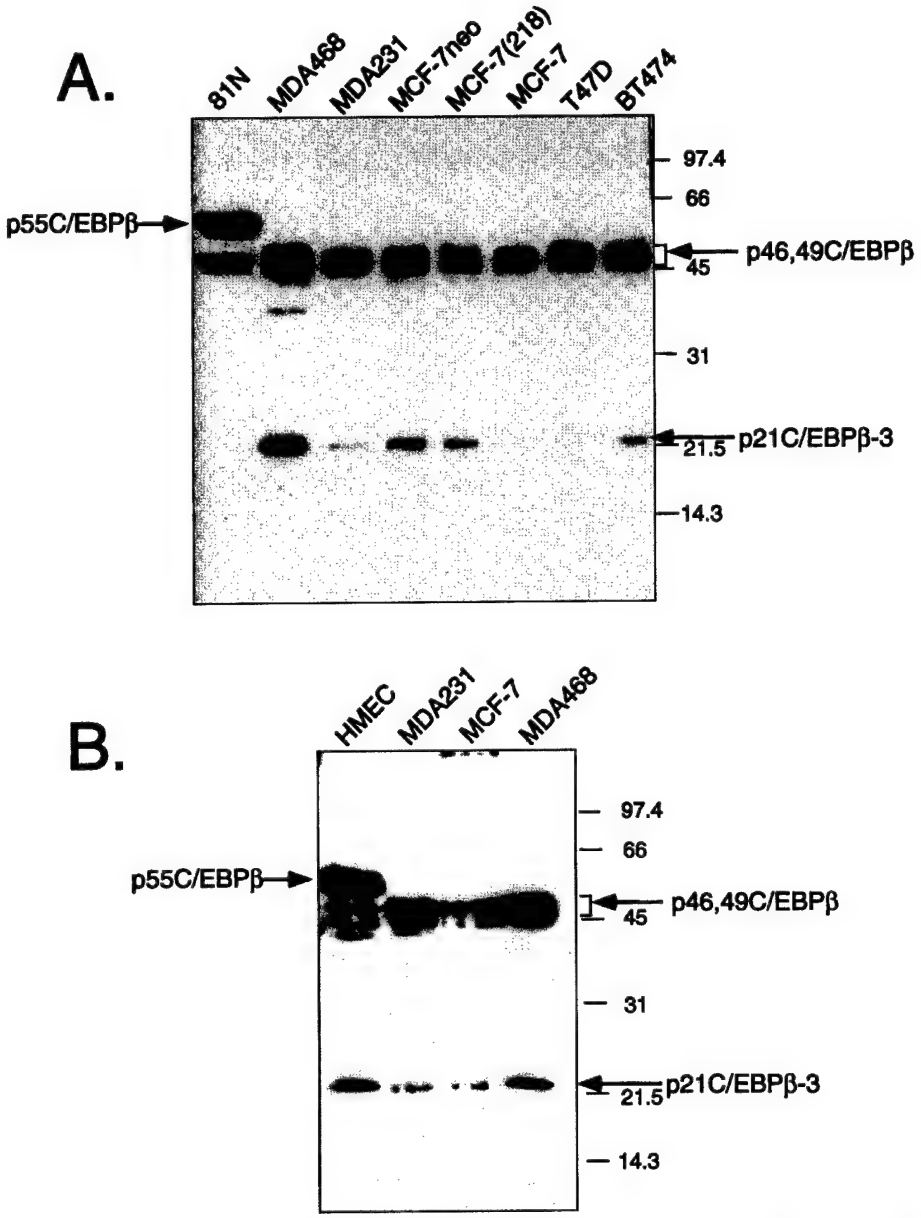


Fig. 2. Breast cancer cell lines display a different pattern of C/EBPβ expression than normal cells. **A:** Whole cell extracts were made from a battery of breast cancer cell lines as well as from a population of normal cells, 81N. Equal amounts of total cell protein were analyzed by SDS-12% polyacrylamide gel electrophoresis. Immunoblotting was performed with C-terminal C/EBPβ antibody (Santa Cruz). **B:** Whole cell extract was prepared from cultured normal human mammary epithelial cells (HMECs) obtained from Clonetics and analyzed in parallel with MDA231, MCF-7, and MDA468 breast cancer cell extracts as in (A).

to immunoblot extracts of transfected or non-transfected NIH 3T3 cells, the antibody reacted with a 58 kD protein present only in the transfected cells (Fig. 3A). To ensure that the 58-kD protein is in fact human C/EBPβ-1, we stripped the blot and reprobed with T7 tag monoclonal antibody because the transfected protein carries a T7 tag. The T7 tag antibody recognized the same 58 kD protein as the N-terminal antibody (Fig. 3A). Thus, we conclude that our N-terminal antibody can recognize human C/EBPβ-1. Moreover, due to the low transfection efficiency of NIH 3T3 cells, 130 μg of total cellular protein was loaded onto each lane of the gel in Figure 3A to enable detection of the transfected protein. While Ponceau S staining confirmed that ample cellular protein was transferred to the filter, the affinity-purified N-terminal antibody did not crossreact appreciably with any other proteins on the Western blot, at least in mouse cells. Interestingly, although the predicted MW of the protein encoded by the human C/EBPβ-1 expression vector is 39 kD including the T7 tag (which adds about 3 kD to the MW), the expressed protein actually migrates at 58–62 kD. Thus, we would expect a full length, untagged human C/EBPβ-1 protein to migrate on our gel system at approximately 55–59 kD, which is

in good agreement with the 55 kD protein observed in the normal mammary epithelial cells in Figure 2.

To determine if our N-terminal antibody would indeed specifically recognize a 55–59 kD protein in normal mammary epithelial cells, we next performed immunoprecipitation experiments using ^{35}S-methionine labeled HMECs (Fig. 3B). The antibody precipitated a protein of approximately 59 kD molecular weight (Fig. 3B, lane 4). This interaction was specific, as determined by beads alone (Fig. 3B, lane 2) and peptide blocking controls (Fig. 3B, lane 3). Finally, we performed Western analyses of normal mammary epithelial cells with the affinity-purified N-terminal antibody. For these experiments, HMEC cells were fractionated into cytoplasmic and crude nuclear fractions and duplicate lanes from the same gel were probed with either the N-terminal or C terminal antibody as indicated in Figure 3C. The N-terminal antibody detected two major bands of 51–58 and 45–48 kD in the crude nuclear fraction prepared from HMECs (Fig. 3C, lane 4). The C-terminal C/EBPβ antibody also detected the 51–58 kD band in a parallel sample of HMEC nuclear fraction (Fig. 3C, lane 2). Because this band (denoted simply p55 in the Figure) is recognized by both the C-terminal and N-terminal C/EBPβ antibodies and has the same MW as the full length human C/EBPβ protein expressed by transient transfection (Fig. 3A), we conclude this protein is C/EBPβ-1. The 45–48 kD protein also detected by the N-terminal antibody in the nuclear HMEC fraction could be a cross-reacting protein because it is not recognized by the C-terminal antibody (lane 2). However, the 45–48 kD protein could also represent a slightly proteolized form of p55 which has lost the C-terminal epitope and is therefore no longer seen by the C-terminal antibody.

In addition to p55 or C/EBPβ-1, the C-terminal antibody also recognized a strong band of 43-45kD and a more minor doublet of 48 and 50kD in the cytoplasmic fraction of HMECs (Fig. 3C, lane 1). Due to their nearly full-length size but lack of recognition by the N-terminal antibody (lane 3), these proteins are likely to be C/EBPβ-2 and modified forms of C/EBPβ-2, respectively. Their cytoplasmic localization will be discussed further in Figure 6. Finally the C-terminal antibody also uniquely detected proteins of 30 and 21 kD which were exclusively nuclear. p30 is likely to represent a proteolytic fragment of a longer C/EBPβ isoform and p21 corresponds to the MW for C/EBPβ-3 or LIP. Whether p21C/EBPβ-3 is generated by alternative translation initiation or proteolysis (artifactual or otherwise) in these cells has not been determined.

In Figure 3, we also examined the expression of C/EBPβ-1 in several cell types in addition to HMECs. As shown in Figure 3C, lane 6, C/EBPβ-1 was not detectable with the N-terminal antibody in a whole cell extract from MCF-7 cells. This is in agreement with Figure 2, where the C-terminal antibody failed to detect a slower migrating 55 kD protein in any of the breast cancer cell lines, including MCF-7. At this time we were also able to obtain mammary ductal epithelial cells (MDEs) which are exfoliated and secreted in human breast milk (Taylor-Papadimitriou et al., 1977; Thompson et al., 1998). Western analysis using the N-terminal antibody showed that MDEs displayed expression of a 55 kD C/EBPβ isoform, C/EBPβ-1 (Fig. 3C, lane 5). A larger 64 kD band was also detected by the N-terminal antibody in the MDEs. Both the major 55 kD band as well as the 64 kD MDE-specific band reacted with the C-terminal antibody (data not shown), suggesting that the larger 65 kD band may be a more highly modified form of C/EBPβ-1. However, due to a lack of material, we were unable to further characterize this band. Based on the data in Figures 2 and 3, we concluded that C/EBPβ-1 is present in normal mammary epithelial cells. C/EBPβ-1 was not detectable in the breast cancer cell lines using either the C terminal (Fig. 2) or the N terminal antibody (Fig. 3).

C/EBPβ-2 expression is absent in normal primary breast tissue

We wanted to investigate whether the difference in expression profiles of C/EBPβ between normal and cancer cells in culture were also seen in primary tissues. Frozen samples of tissue taken from reduction mammoplasties and from breast tumors were obtained from the Human Tissue Acquisition Core at Vanderbilt University Medical Center. Whole cell extracts were prepared from the tissue samples and analyzed by immunoblotting using the C-terminal C/EBPβ antibody. As shown in Figure 4A, a single isoform of C/EBPβ at approximately 55 kD was detected with the C-terminal antibody in 12 normal tissue samples obtained from reduction mammoplasties (Fig. 4A, left panel). The N-terminal antibody also detected the same size band (Fig. 4A, right panel), confirming that this isoform is C/EBPβ-1, since only the full length protein could be recognized by the N-terminal antibody. Interestingly, when ten primary human breast cancer tissue samples were analyzed by immunoblotting, seven of the samples contained an additional C/EBPβ protein doublet at 45–48 kD when probed with the C-terminal antibody (Fig. 4B, left panel). The 45–48 kD doublet was particularly abundant in six of the ten samples (T1, T3, and T6–9). On a duplicate blot, the N-terminal antibody failed to detect the 45–48 kD doublet, although a 55 kD species was present at some level in all samples (Fig. 4B, right panel). Because the 45–48 kD doublet is recognized by the C-terminal, but not N-terminal antibody (Fig. 4B, right panel), it is very likely C/EBPβ-2. The differential expression of C/EBPβ-2 in tumor but not normal tissue is further documented in Figure 5, which is another immunoblot of six of the tumor samples and three of the normal tissue samples directly compared on the same blot. In this darker exposure, minor levels of C/EBPβ-2 expression are in fact observed in T4, and also possibly T2 and T5, with the C-terminal antibody in Figure 5A which cannot be detected in Figure 4B. However, absolutely no C/EBPβ-2 bands are present in the three normal samples (Fig. 5A, lanes 2–4). In Figure 5B, the blot in 5A was stripped and reprobed with the N-terminal antibody. A 55kD band was detected by the N-terminal antibody in all samples, while the 45–48 kD protein was not detected. [Note: the gaps in the bands in T5 and T6 are artifactual, as a 55 kD protein was clearly detected in these samples by the N-terminal antibody in Fig. 4B, see left panel].

We conclude that all normal tissue samples express exclusively C/EBPβ-1, whereas seven of ten tumor samples had acquired significant C/EBPβ-2 expression. The tumor samples also contained C/EBPβ-1; however,

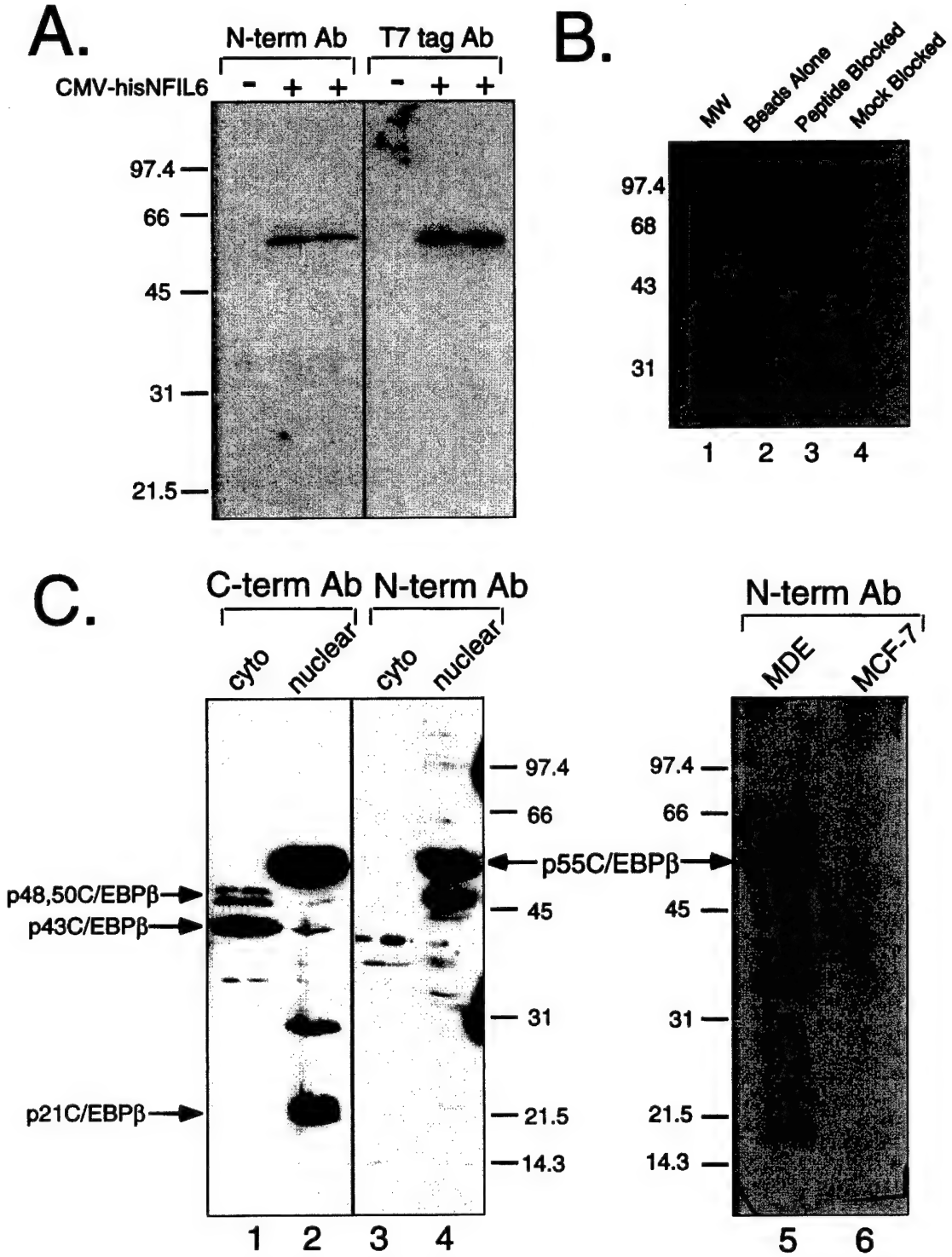


Fig. 3. Characterization of the N-terminal human C/EBPβ-1 antibody. **A:** NIH 3T3 cells were transfected with 8 μg of a CMV-driven his-tagged NFIL6 expression vector modified so that only protein initiating at the first ATG would be produced (+) or control CMV-4 vector (−). Cells were harvested 40 h post-transfection and whole cell extracts prepared and analyzed as in Figure 2. Immunoblotting was performed with the N-terminal C/EBPβ or an antibody to the T7 histidine tag (Novagen). **B:** ^{35}S methionine-labeled whole cell extracts from HMEC cells were incubated with agarose beads alone (**lane 2**), beads plus N-terminal antibody (**lane 3**), or beads plus N- terminal antibody pre-blocked with 700 ng of N-terminal peptide (**lane 4**). Proteins bound to the beads were analyzed by SDS-12% polyacrylamide gel electrophoresis. Autoradiography of the dried gel is shown. **C:** HMECs were fractionated by Triton lysis into a cytoplasmic (**lanes1, 3**) and nuclear (lanes 2, 4) fraction. Equal proportions of these fractions were analyzed in duplicate by SDS-12% polyacrylamide gel electrophoresis and immunoblotting was performed with either the C-terminal or N-terminal C/EBPβ as indicated. In **lanes 5** and **6**, whole cell extracts from mammary epithelial cells exfoliated in human breast milk (MDE) or from the MCF-7 human breast cancer cell line were analyzed by SDS-12% polyacrylamide gel electrophoresis and immunoblotting with N-terminal C/EBPβ antibody.

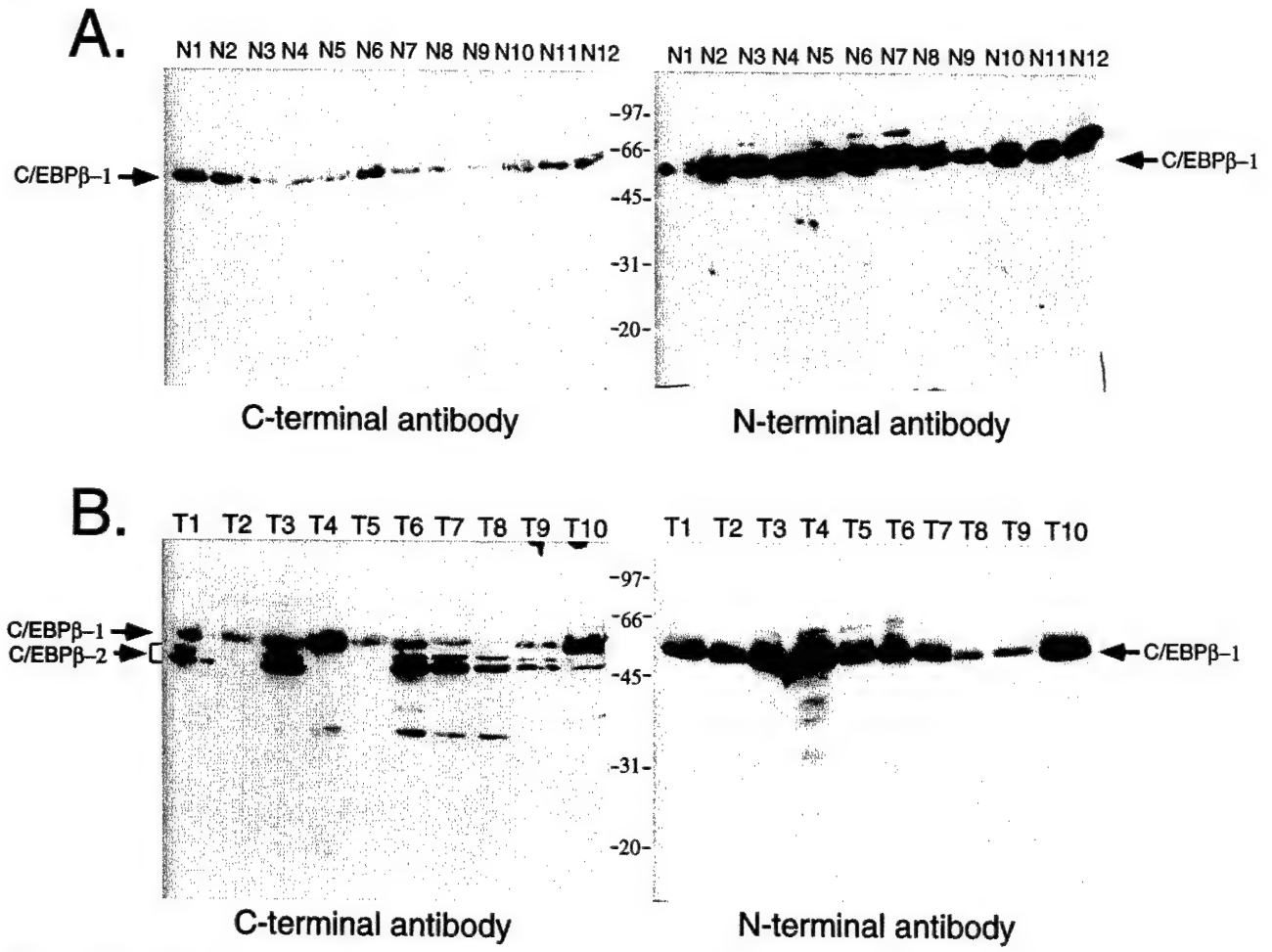
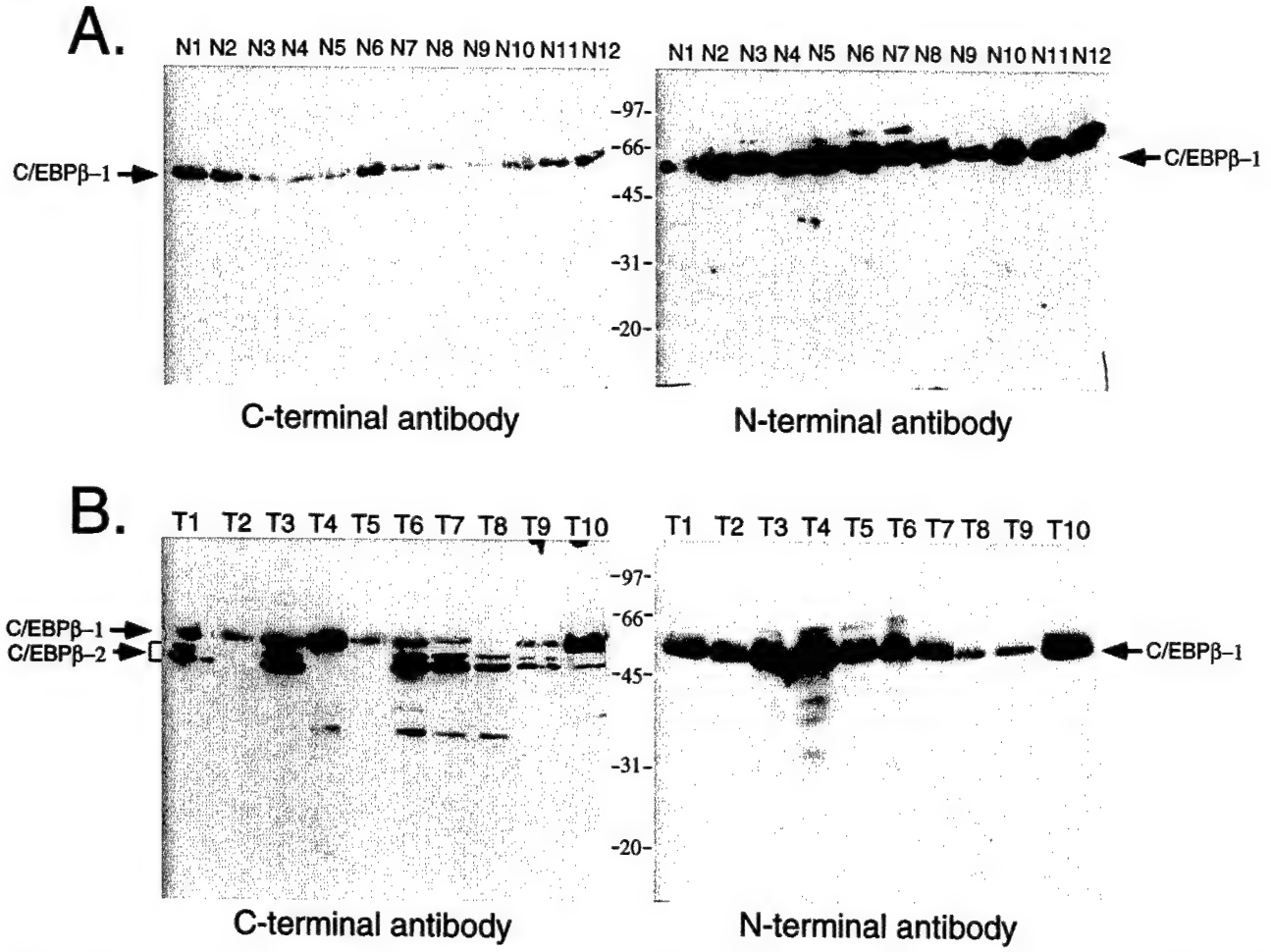


Fig. 4. Primary human breast tumors acquire C/EBPβ-2 expression. Whole cell extracts were prepared from normal breast tissue from reductive mammoplasty (**A**) or breast tumors (**B**) obtained from the Tissue Procurement Core at Vanderbilt. Equal amounts of total cell protein were analyzed by SDS-12% polyacrylamide gel electrophoresis. Duplicate gels were subject to immunoblotting with either the C-terminal or N-terminal C/EBPβ as indicated

these samples were comprised of both tumor and normal tissue. Using this analysis, it is impossible to determine in which cells, tumor or normal, the C/EBPβ-1 is expressed. Therefore, it is possible that the C/EBPβ-1 we are detecting is expressed in the surrounding normal tissue. Although the number of tumor samples available to us for analysis was small, the incidence of significant C/EBPβ-2 expression in seven of ten samples compared to zero of 12 normal samples results in a *P*-value of 0.0007 (less than 0.05 in Fisher's exact test) which is statistically significant. Moreover, it is interesting to note that of the ten tumors examined here, seven were high grade, invasive mammary carcinomas, and all seven showed significant C/EBPβ-2 expression. T4, which showed intermediate expression, and T2 were invasive, but of intermediate and low grade, respectively, with T2 being primarily ductal carcinoma in situ. T5 was an encysted male breast tumor with papillary features. However, because the primary tissue samples differ in the percent which is tumor mass, it is difficult to perform quantitative, comparative studies of C/EBPβ-2 expression by immunoblotting.

Breast cancer cell lines display altered subcellular distribution of C/EBPβ- 2

Our next step was to investigate the subcellular partitioning of the C/EBPβ isoforms within the normal and cancer cell lines. Immunoblotting was performed on cytoplasmic and nuclear extracts from breast cancer cell lines and normal cell lines. We had previously found that normal HMECs restricted the majority of their C/EBPβ-2 to the cytoplasm (Fig. 3C). This is again the case in Figure 6 (compare lanes 1 and 2) with an isolate of HMECs different from that used in Figure 3. In contrast, cytoplasmic and nuclear extracts prepared in parallel from the breast cancer cell line, MDA 231, and normal, but immortalized MCF 10As, contained much higher levels of C/EBPβ-2 in the nucleus (Fig. 6, compares lanes 2, 4, and 6). The C/EBPβ-2 in the nucleus of the tumor cells (lane 6) also appears to be more modified. Given that multiple kinases are known to phosphorylate C/EBPβ, it is not unlikely that the more slowly migrating bands represent increased phosphorylation of C/EBPβ-2. The N terminal antibody was used to confirm the



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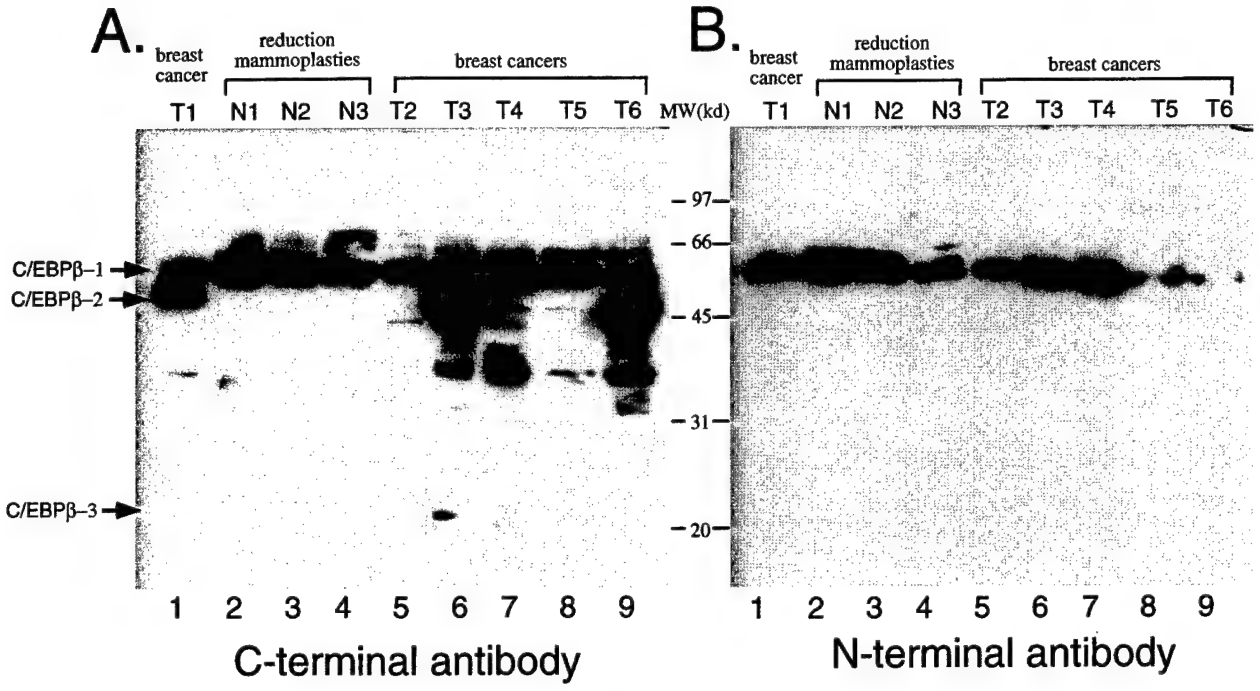

Fig. 5. Normal breast tissue from reductive mammoplasty expresses only C/EBPβ-1. **A:** Equal amounts of total cell protein from six primary breast tumors (T1–T6) or three normal breast tissue samples from reductive mammoplasty (N1-N3) were analyzed by SDS-12% polyacrylamide gel electrophoresis follwed by immunoblotting with C-terminal C/EBPβ antibody. **B:** The immunoblot in (A) was stripped and reprobed with N-terminal C/EBPβ antibody. [The blot in (A) was overexposed to show that even with longer exposures, the C-terminal antibody picks up no C/EBPβ-2 in the normal samples].

identity of C/EBPβ-1(data not shown). The C/EBPβ-1 isoform, present only in the normal cells examined, was strictly nuclear as was the C/EBPβ- 3 isoform (see Fig. 3C).

C/EBPβ- 2, but not C/EBPβ- 1, will activate the cyclin D1 promoter

Given their distinct expression profiles, we next wanted to determine if there are functional differences between C/EBPβ-1 and C/EBPβ-2. We examined the activity of C/EBPβ-1 and C/EBPβ-2 at a promoter relevant in breast cancer, cyclin D1. Based on sequence analysis, we identified several putative C/EBPβ sites in the cyclin D1 promoter at −970, −652, −559, −463, and −209. Oligonucleotides representing the five possible sites were radiolabeled and used in EMSAs along with nuclear extracts from the breast cancer cell line MDA 231(see Fig. 7). One site at −559 bound C/EBPβ from the extract, as determined by antibody supershift analysis. From previous work in our laboratory, we suspect that the multiple complexes detected represent homo- and heterodimers of C/EBPβ-2 and C/EBPβ-3, the two forms of C/EBPβ detected in MDA 231 cells.

We used PCR to clone a 1.2 kB fragment of the cyclin D1 promoter from the MDA 231 cell line. This fragment

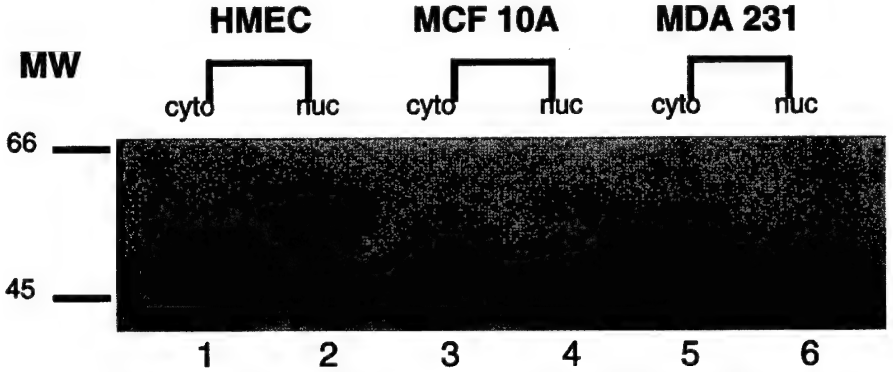

Fig. 6. Nuclear/cytoplasmic distribution of C/EBPβ isoforms in normal and transformed human mammary epithelial cells. Crude nuclear and cytoplasmic extracts were prepared by Triton lysis from HMECs (**lanes 1, 2**), MCF-10A cells (**lanes 3, 4**) or MDA231 (**lanes 5, 6**) breast cancer cells. Equal proportions of nuclear and cytoplasmic extracts were loaded onto a 12% SDS gel transferred to Immobilon-P and probed with C-terminal C/EBPβ antibody.

was then subcloned into a luciferase reporter vector, pGL3. Using transient transfections, we determined that all three forms of C/EBPβ would regulate the cyclin D1 promoter. In both NIH 3T3 cells and HMECs, C/EBPβ-2 activated the cyclin D1 promoter (Fig. 8A). Strikingly, C/EBPβ-1, the activator isoform found primarily in the normal non-proliferative breast cells failed to activate, and indeed slightly repressed, the cyclin D1 promoter in both of the cell lines tested. In Figure 8B, the C/EBPβ-1 construct was tested using another promoter regulated by C/EBPβ, the Rous sarcoma virus (RSV) LTR, to insure that it does encode a functional activator protein. The activity of C/EBPβ-3 at the cyclin D1 promoter was also examined. This protein caused substantial repression of the cyclin D1 promoter (data not shown).

C/EBPβ-1 will bind DNA

As a first step toward explaining the difference in the ability of the C/EBPβ activator isoforms to activate, we determined whether or not C/EBPβ-1 would bind to DNA and the cyclin D1 promoter in particular. Nuclear and cytoplasmic extracts from HMECs were run on adjacent lanes of a SDS gel and transferred to an Immobilon filter. While parallel lanes of nuclear and cytoplasmic extracts were processed for Western analysis, a lane containing nuclear extract was cut into 3-mm slices. These slices were placed into elution solution to recover DNA binding activity. The Immobilon slices representing approximately 97–25 kD were used in gel shifts along with a ^{32}P labeled oligonucleotide representing the C/EBPβ site at −221 in the human beta-casein promoter or the −559 site in the cyclin D1 promoter. Similar results were obtained with either DNA as shown in Figure 9. Binding to both DNA probes was observed from fractions 7 and 8 corresponding to the migration of C/EBPβ-1, as confirmed by the Western analysis. Binding was also recovered from fractions 10 and 11, corresponding to the migration of C/EBPβ-2. Given the low amount of material recovered from the

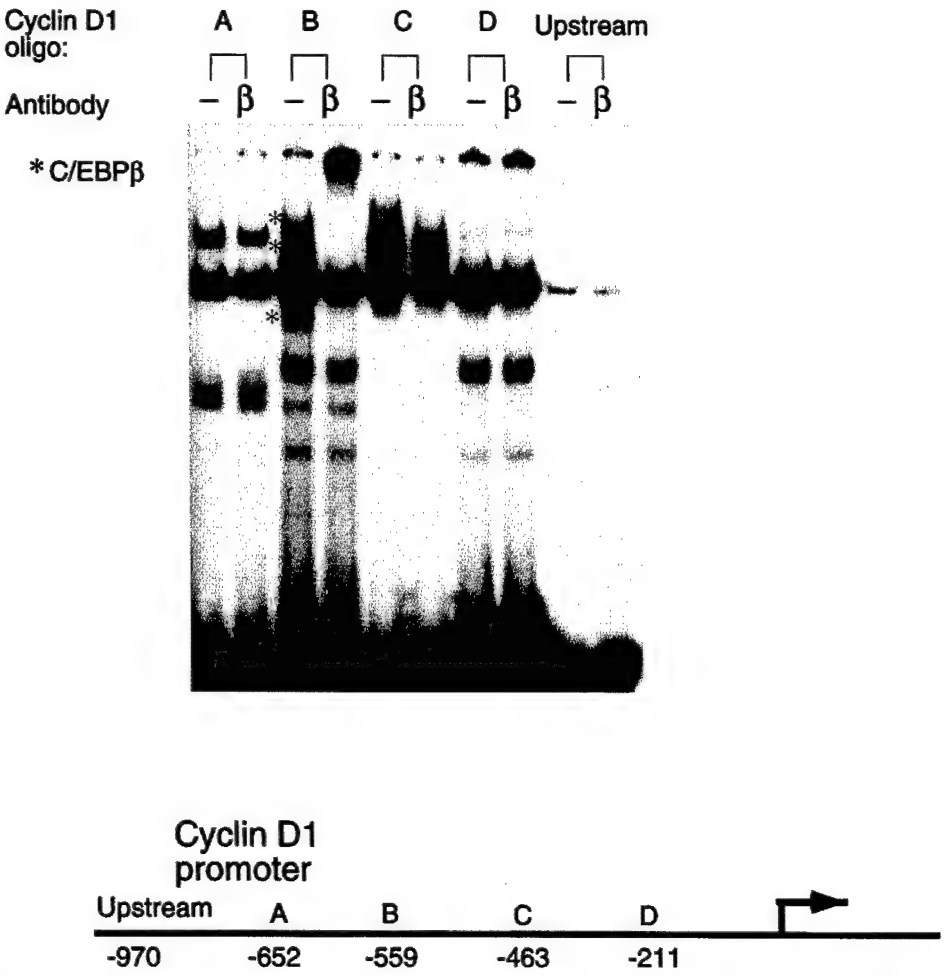

Fig. 7. C/EBPβ will bind the −559 site in the cyclin D1 promoter. Radiolabeled oligonucleotides representing potential C/EBPβ binding sites in the cyclin D1 promoter as depicted in the diagram were incubated with either nuclear extracts from MDA 231 cells or extracts pre-mixed with C-terminal C/EBPβ antibody. Reactions were analyzed by gel electrophoresis and the dried gel was exposed to film. C/EBPβ protein-DNA complexes in the autoradiograph are starred. The DNA from the upstream site was older and therefore less radioactive than the other four oligonucleotides.

immobilon slices, it is not possible to carry out competition analyses to demonstrate the binding specificity of fraction #7. However, the binding activity in fraction #7 is diminished and supershifted by C-terminal C/EBPβ antibodies, confirming that the DNA binding activity is a 55 kD C/EBPβ isoform or C/EBPβ-1. We conclude that the failure of C/EBPβ-1 to activate the cyclin D1 promoter is not due to a failure to bind DNA. A more complicated scenario such as the inability of C/EBPβ-1 to form necessary protein/protein interactions is likely responsible for the inability of this protein to activate this promoter (see Discussion).

DISCUSSION

C/EBPβ is a widely expressed transcription factor that can exist as three different isoforms, the full length protein, C/EBPβ-1, a 23 amino acid N-terminally truncated protein, C/EBPβ- 2, and a protein with a large N-terminal truncation, C/EBPβ-3. Using a new antibody specific for the N-terminal amino acids unique to human C/EBPβ-1, we uncovered substantial differences in the expression of C/EBPβ-1 and -2 in normal and transformed mammary epithelial cells. C/EBPβ-2 was not detected in normal, mostly non-dividing tissue from reduction mammoplasties, which expressed only C/EBPβ-1. Abundant C/EBPβ-1 expression was also detected in secretory mammary epithelial cells exfoliated in human breast milk, where an additional, potentially more highly modified form of C/EBPβ-1 was also observed. In contrast, a majority of primary human breast tumors examined had acquired significant levels of C/EBPβ-2 expression. C/EBPβ-2 was also the principal isoform expressed in all cultured breast cancer cell lines we examined; none of these tumor cell

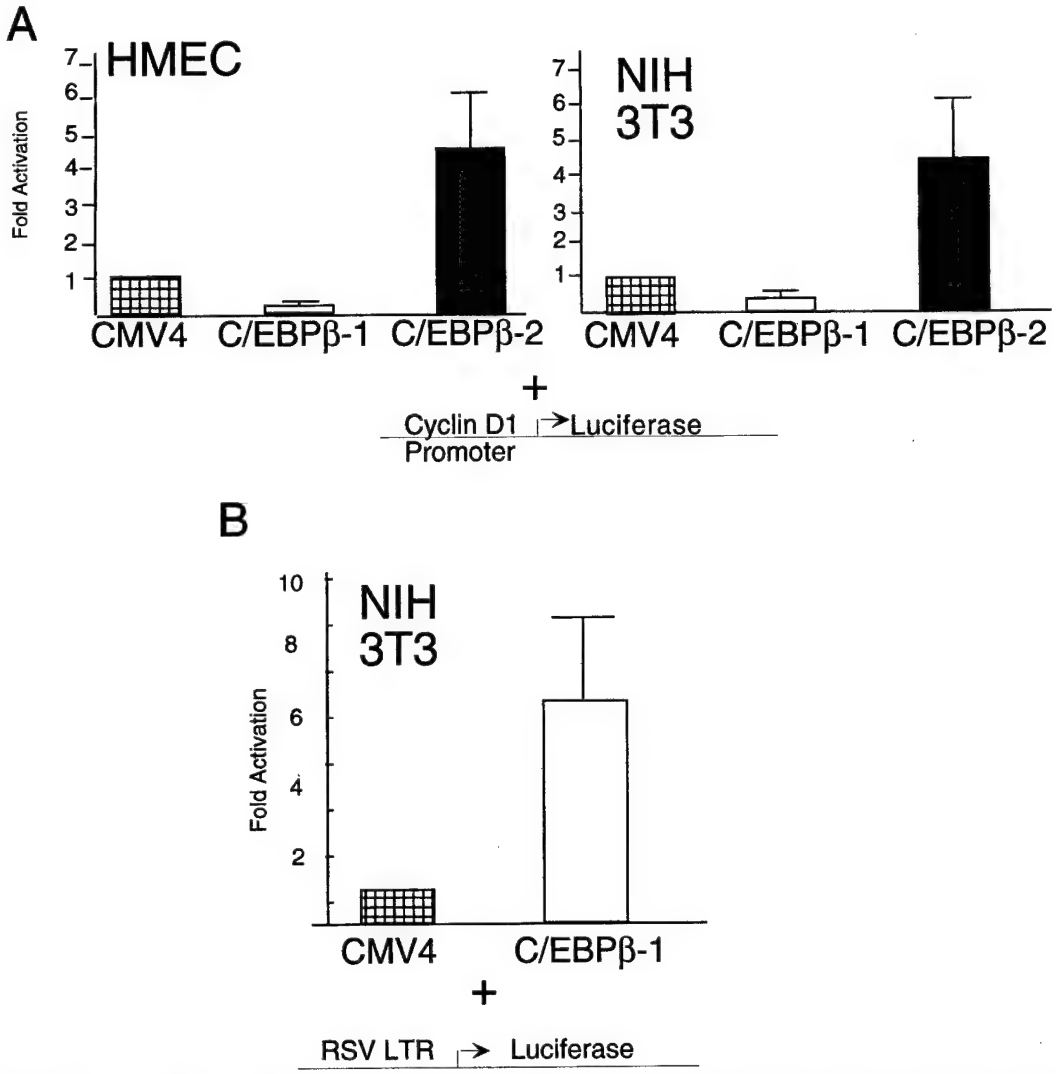

Fig. 8. C/EBPβ-2, but not C/EBPβ-1, will activate the cyclin D1 promoter. **A:** HMECs were transfected for 5 h with 2 μg of the cyclin D1/ luciferase construct (pGL3cycD) and 2 μg of empty vector (CMV4) or 2 μg of expression vector for either C/EBPβ-1 or C/EBPβ-2 using Geneporter liposomes. NIH 3T3 cells were transfected for 8 h with the same DNAs using Novafector liposomes. Data are the average of three separate experiments with duplicate samples. **B:** NIH 3T3 cells were transfected as in (A) except that calcium phosphate precipitation was used and samples contained 100 ng of an RSV LTR luciferase reporter gene.

lines expressed C/EBPβ-1. Interestingly, we obtained similar results when we examined other tissues, namely prostate and colon. That is to say, C/EBPβ-2 is the major isoform in prostate and colon carcinoma cell lines, whereas samples of normal, primary prostate, and colon tissue express C/EBPβ-1 exclusively.

It is important to note that the analysis of primary breast tumors and normal breast tissue was not meant to be a clinical study but to demonstrate that C/EBPβ-2 expression is not an idiosyncracy of breast cancer cell lines. Because C/EBPβ-1 was also detected by immunoblotting in the primary breast tumor samples, lack of C/EBPβ-1 in the breast tumor cell lines could reflect an adaptation to prolonged growth in culture. However, because the primary breast tumor samples were heterogeneous in nature consisting of both normal and tumor tissue, we cannot exclude the possibility that the C/EBPβ-1 detected in the immunoblots actually arises from the surrounding normal and not tumor tissue. In situ immunohistochemical analyses with the N-terminal antibody will be necessary to resolve this issue, studies we are currently pursuing.

Although C/EBPβ-1 and -2 are both transactivators, their distinct expression profiles suggested that functional differences might exist between the two isoforms. Because significant C/EBPβ-2 expression had been acquired by seven of ten human breast tumors and this was the only C/EBPβ activator isoform expressed in the cultured breast tumor cell lines, we tested whether C/EBPβ-2 could transactivate the promoter of the cyclin D1 gene in transient cotransfection experiments. Indeed, transactivation was observed by C/EBPβ-2 but not C/EBPβ-1. While this work was in progress Kowenz-Leutz and Leutz (1999) reported on functional differences between rat C/EBPβ-1 and -2 (called LAP* and LAP, respectively in their work), demonstrating that only C/EBPβ-1, but not C/EBPβ-2, can cooperate with Myb to activate the differentiation specific *mim-1* gene in chromatin. This specificity is likely due to the interaction of the N-terminal amino acids of LAP*, or C/EBPβ-1, with the SWI/SNF chromatin remodeling complex. Kowenz-Leutz and Leutz did not observe functional differences between LAP* and LAP in transient transfection assays unless the cell line was

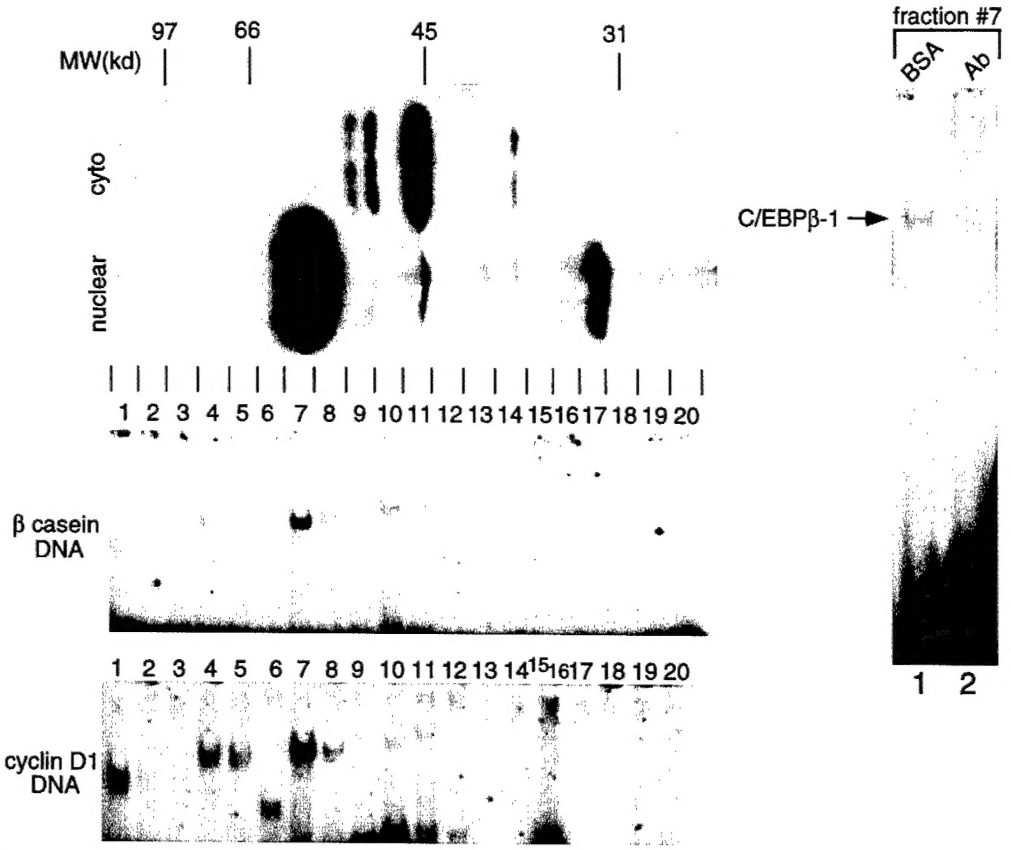

Fig. 9. C/EBPβ-1 will bind DNA. Western blot of cytoplasmic and nuclear extracts from HMECs probed with C-terminal C/EBPβ antibody is shown in the top panel. A parallel lane of the filter containing nuclear extract was cut into 3-mm slices and proteins eluted from slices representing approximately 97–25 kD were used in EMSAs along with ^{32}P labeled DNAs representing the C/EBPβ sites in the beta-casein promoter (middle panel) or the −559 site in the cyclin D1 promoter (lower panel); along with DNA binding assays were run on adjacent lanes of an SDS gel and transferred to Immobilon. The DNA binding assay was repeated with fraction #7 (corresponding to the apparent MW of C/EBPβ-1) and beta-casein DNA except that fraction #7 was preincubated with C-terminal C/EBPβ antibody (**lane 2**) or BSA (**lane 1**) prior to the addition of radiolabeled DNA.

deficient in SWI/SNF functions. Our results extend their observations by showing that functional differences between C/EBPβ-1 and -2 can also be observed in transient transfection assays with certain promoters, such as cyclin D1. The transactivation domain of C/EBPβ common to both C/EBPβ-1 and -2 in the N-terminal half of the protein is known to interact with other factors involved in transcriptional activation such as TBP, TFIIB, and the coactivator and histone acetylase CBP/p300 (Nerlov and Ziff, 1995; Mink et al., 1997). Although we do not know which factors C/EBPβ-2 is interacting with to transactivate the cyclin D1 promoter, it is possible that the additional amino acids of C/EBPβ-1 block this interaction, either sterically, or by interacting with another distinct set of factors such as the SWI-SNF chromatin remodeling complex.

The ability of C/EBPβ-1 to interact with the SWI-SNF chromatin remodeling complex supports our proposal that C/EBPβ-1 regulates differentiation-specific genes and C/EBPβ-2 targets growth-promoting genes. While there may be some genes that are transactivated equally well by both C/EBPβ-1 and -2, the commitment of cells to a differentiation pathway requires the activation of transcriptionally-silent genes in chromatin and thus a transcription factor capable of overcoming the repressive effects of chromatin. Alveolar development and differentiation of mammary epithelial cells is severely impaired in pregnant C/EBPβ-null mice (Robinson et al., 1998; Seagroves et al., 1998). The differentiation program leading to a functional gland is not executed, as the expression of milk proteins such as WAP, WNM1, and β-casein is undetectable in C/EBPβ-null mice at term (Robinson et al., 1998; Seagroves et al., 1998). The sole expression of C/EBPβ-1 in normal, mostly non-dividing mammary tissue, as well as the presence of a more slowly migrating, possibly more modified form of C/EBPβ-1 in secretory mammary epithelial cells that have been actively expressing milk proteins strongly suggests that C/EBPβ-1 is involved in transactivating the promoters of the milk protein genes either directly (such as β-casein) or indirectly. During the differentiation of other cell types, C/EBPβ acts in a combinatorial fashion with other transcription factors. For example, C/EBPβ plus Myb leads to differentiation along the myeloid lineage (Burk et al., 1993; Ness et al., 1993), whereas C/EBPβ collaborates with the transcription factor PPARγ to specify adipogenic differentiation (Wu et al., 1995). Whether C/EBPβ-1 collaborates with another transcription factor(s) in the early stages of mammary epithelial differentiation remains to be determined.

Evidence has recently been accumulating that C/EBPβ plays an important role in cellular proliferation during liver regeneration. Following partial hepatectomy, mice lacking the C/EBPβ gene display impaired liver regeneration (Greenbaum et al., 1998) and C/EBPβ $^{-/-}$ mouse hepatocytes are refractory to the growth-promoting effects of TGFα(Buck et al., 1999). The latter defect can be rescued by re-expression of C/EBPβ in the C/EBPβ $^{-/-}$ cells. We have shown that C/EBPβ is involved in activating the serum response element in the promoter of an early response gene, c *fos*, by forming a complex with serum response factor (Hanlon and Sealy, 1999). C/EBPβ is required for the proliferation of mammary epithelial cells (Robinson et al., 1998; Seagroves et al., 1998), and our data indicate that C/EBPβ-2 is likely to be the responsible isoform. It is the only activator isoform of C/EBPβ expressed in the breast cancer cell lines we examined, it is capable of activating the cyclin D1 promoter, and C/EBPβ-2 expression is acquired by a majority of primary breast tumors we examined.

Although tissue from reduction mammoplasties lacks C/EBPβ-2 expression, mammary epithelial cells from reduction mammoplasties (HMECs) placed in a rich growth medium in culture acquire C/EBPβ-2 expression. However, C/EBPβ-2 expressed in normal mammary epithelial cells (HMECs) is primarily cytoplasmic. Breast cancer cells display a much higher proportion of C/EBPβ-2 in the nucleus. Since little is known about the mechanism(s) regulating cytoplasmic to nuclear transport of C/EBPβ-2, it is not clear how growth-promoting signals influence the proportion of C/EBPβ-2 in the nucleus. Any mechanism must also explain why C/EBPβ-2 can be sequestered in the cytoplasm whereas C/EBPβ-1, with its 21–23 amino acid N-terminal extension, appears to be constitutively nuclear.

Also of note is the fact that nuclear C/EBPβ is more highly modified in the breast cancer cells, compared with the nuclear C/EBPβ of the primary HMECs. C/EBPβ is the target of several mitogenic signaling pathways, including some of interest in the context of breast cancer. Activated Ras causes phosphorylation of C/EBPβ at Thr 235 via the MAP kinase pathway in vivo and in vitro (Nakajima et al., 1993; Hanlon and Sealy, 1999). At least one consequence of this phosphorylation is potentiation of the transactivation by C/EBPβ of the *cfos* serum response element (Hanlon and Sealy, 1999). Serum response factor (SRF) recruits C/EBPβ to the SRE; however, C/EBPβ and SRF interact only upon Ras-dependent phosphorylation of C/EBPβ by ERK2 and p90Rsk2. Work in our laboratory has indicated that C/EBPβ is a target of the PI3Kinase pathway as well (Hanlon et al, manuscript submitted). Both ras and PI3Kinase are among the targets of erbB-2 signaling (reviewed in Reese and Slamon, 1997); studies evaluating the effects of erbB-2 upon C/EBPβ, paticularly C/EBPβ-2, will prove interesting.

In this work, we also observed differences in the expression of C/EBPβ-3 among the normal mammary epithelial and breast tumor cells that we studied. Zahnow et al. (1997) detected C/EBPβ-3 (also known as LIP) overexpression in a subset of infiltrating ductal carcinoma specimens. They propose that C/EBPβ-3 expression is oncogenic (Zahnow et al., 1997). In this study, seven of the ten tumor specimens examined were considered high grade infiltrating mammary carcinomas; however, none expressed any appreciable level of C/EBPβ-3. C/EBPβ-3 was detected in some of the breast cancer cell lines (Fig. 2) although generally at low levels with the exception of the MDA 468 cells. However, even higher levels of C/EBPβ-3 than present in the MDA 468 cells were detected in HMECs growing in culture (see Fig. 3) and these cells are neither transformed nor immortalized. Thus, there does not appear to be a strong correlation between high level of C/EBPβ-3 expression and transformation of mammary epithelial cells. Unfor-

tunately, analysis of C/EBPβ-3 levels in cells is complicated by the propensity of the longer forms of C/EBPβ to undergo proteolytic degradation during in vitro manipulations of cells or tissues (Baer and Johnson, 2000). Extreme care to prevent adventitious proteolysis is required before meaningful conclusions about C/EBPβ-3 levels in cells can be obtained.

The N-terminal antibody we describe here has been a valuable tool to document differences in expression of C/EBPβ-1 and -2 in normal vs. transformed mammary epithelial cells. That these differences have functional significance is supported by our preliminary experiments with the cyclin D1 promoter. Whether C/EBPβ-2 promotes growth of mammary epithelial cells by upregulating cyclin D1 expression will require additional studies in which the consequences of increased C/EBPβ-2 expression in the nucleus of normal mammary epithelial cells is addressed. This may require deciphering the mechanisms by which normal mammary epithelial cells sequester C/EBPβ-2 in the cytoplasm, a feature apparently lost in tumor cells. Although we selected the cyclin D1 gene because it has been strongly implicated in the pathogenesis of breast cancer, there are likely to be many other genes differentially regulated by C/EBPβ-1 vs. C/EBPβ-2 in mammary epithelial cells. The emerging microarray technology should be extremely useful for capturing on a genomic scale the functional differences in gene regulation by C/EBPβ-1 and -2 in mammary epithelial cells.

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